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Non-integrin cell adhesion triggers ligand-independent integrin signaling

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LIST OF PUBLICATIONS

uPAR-induced cell adhesion and migration: vitronectin provides the key.

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Ligand-independent adhesion signaling by integrins.

Gian Maria Sarra Ferraris, Carsten Schulte, Chris Denis Madsen, and Nicolai Sidenius

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CONTRIBUTION OF THE SUBMITTED THESIS

Nicolai Sidenius performed all the cloning and the purification of the VN substrates

Carsten Schulte performed the imaging experiments and quantification of cell area of (Fig. 2, 8, 12, 15, 16, 25)

Chris Madsen performed some of the western blot experiments of Fig. 11 and 20 and performed time lapse and phase contrast microscopy of Fig. 2, 3 and 12.

LIST OF ABBREVIATIONS

293 cells: human embryonic kidney 293 cell line
ATF: amino-terminal fragment of uPA
Cas: Crk-associated substrate
CHO cells: Chinese hamster ovary cells
Col: collagen type-1
DI: domain 1 of uPAR
DII: domain 2 of uPAR
DIII: domain 3 of uPAR
DIIDIII: uPAR fragment composed of domains 2 and 3
DDR: discoidin domain receptor
DMEM: Dulbecco's Modified Eagle's Medium
DMSO: dimethyl sulfoxide
ECM: extracellular matrix
EGFR: epidermal growth factor receptor
ERK1/2: extracellular signal-regulated kinases 1 and 2
F-actin: filamentous actin
FA: focal adhesion
FACS: fluorescence activated cell sorter
FAK: focal adhesion kinase
FERM: Band 4.1, Ezrin, Radixin, Moesin
FN: fibronectin
FRET: Föster resonance energy transfer
GAP: GTPase-activating protein
GEF: Guanine nucleotide exchange factor
ICAP-1: Integrin cytoplasmic domain-associated protein 1
ILK: Integrin-linked kinase
GPI: glycosyl-phosphatidylinositol
Ln: laminin
GFP: green fluorescent protein
MAPK: Mitogen-activated protein kinase
MMP: matrix metalloprotease
NCAM: neural cell adhesion molecule
p130Cas: Crk-associated substrate (molecular weight: 130 kDa)
PA: plasminogen activation
PAI-1: plasminogen activator inhibitor type-1
PAI-1/GPI: plasminogen activator inhibitor type-1 containing a GPI-anchor
PBS: phosphate-buffered saline
PH: Plextrin Homology
PI3K: Phosphatidylinositol-3-kinase
PIP2: PI(4,5)P2 or phosphatidylinositol-4,5-bisphosphate
PIP3: PI(3,4,5)P2 or phosphatidylinositol-3,4,5-trisphosphate
PKB/Akt: Protein kinase B / Akt
PKC: Protein kinase C
PTB: Protein tyrosine binding
RGD-motif: three residues (^{47}RGD) within the VN responsible for integrin binding

SMB: somatomedin B domain
suPAR: soluble urokinase-type plasminogen activator receptor
uPA: urokinase-type plasminogen activator
uPAR: urokinase-type plasminogen activator receptor
VN: vitronectin

ABSTRACT

Integrins are the major family of cell surface adhesion receptors responsible for the regulation of the physical contact and biochemical communication between the cell and the surrounding extracellular matrix (ECM). Binding of the extracellular domains of integrins to components in the ECM triggers a series of molecular events commonly referred to as "outside-in" signaling, leading to context-dependent changes in cell morphology, migration and proliferation. In this prevailing paradigm of cell adhesion induced signaling the primary functions of the integrin is to provide the physical transmembrane bridge connecting the intracellular signaling machinery and cytoskeleton to the extracellular environment.

We now present evidence that most, if not all, cell adhesion receptors trigger integrin-dependent outside-in signaling independently of direct contacts between the integrins and their ligands in the ECM. The urokinase-type plasminogen activator receptor (uPAR/CD87) is a non-integrin vitronectin (VN) cell adhesion receptor linked to the outer membrane leaflet by a GPI-anchor. Through an extensive structure-function analysis of uPAR, VN, β1 and β3 we document that cell adhesion induced by the uPAR/VN-interaction triggers integrin-mediated, but ligand independent, cell spreading and signaling. This signaling is fully active on VN lacking functional integrin binding sites and by integrin mutants deficient in ligand binding, but is crucially dependent on an "active" conformation of the integrin as well as its binding to intracellular adaptor proteins including talin and kindlin. This novel paradigm of ligand-independent integrin signaling is not restricted to uPAR as it poses no identifiable constraints to the adhesion receptor with respect to ternary-structure, ligand type or means of membrane anchorage. In full accordance with a general validity of this paradigm, we show that cell adhesion physically

mediated by a signaling-incompetent β3 integrin is effectively translated into cell spreading and signaling by the β1 integrin.

Our results show that integrins are active in transducing adhesion-induced signaling in the absence of their cognate ligands, suggesting that the bi-directional signaling capability of these receptors may have evolved primarily to allow for tightly regulated inside-out signaling.

INTRODUCTION

INTEGRINS

Integrins are transmembrane glycoproteins and constitutes the main family of adhesion receptors expressed on the cell surface. Their name derives from their importance in the structural integrity of cells and tissues. Moreover they form an integral membrane complex that connects the environment outside the cells with the interior cytoskeleton across the plasma membrane.

Integrin receptors are heterodimeric non-covalently bound receptors formed by one α and one β subunit. Today, 18 α and 8 β subunits have been described in mammals and together they form 24 known receptors (Fig. 1).

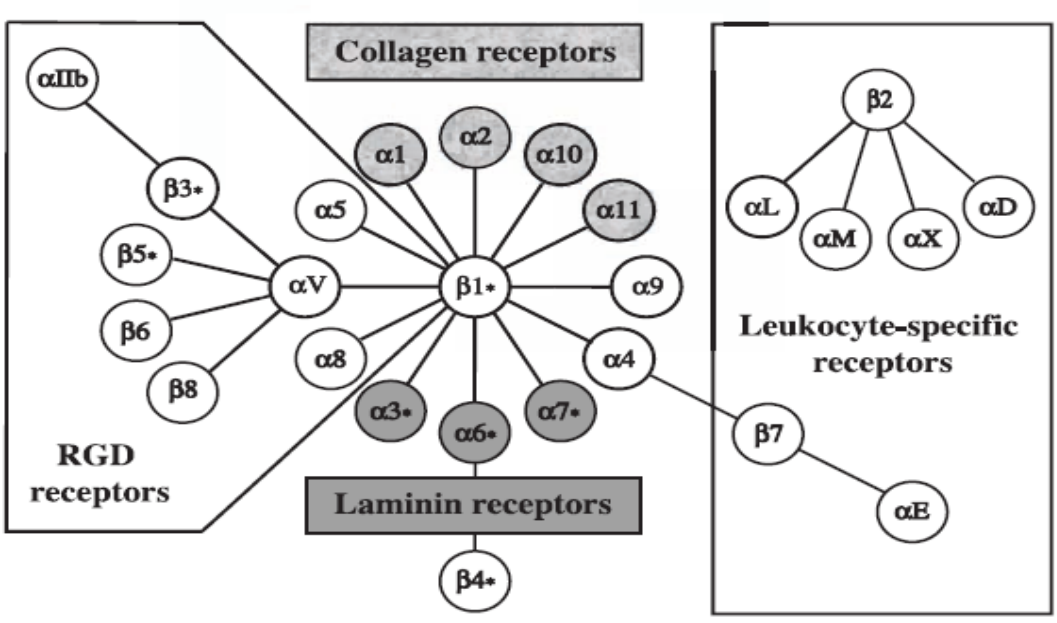


Figure 1 Schematic representation of mammalians integrin array. Integrins are divided according to their ligand specificity and their expression in blood cell types. Every connection between the circles represents a particular integrin heterodimer (adapted from (Hynes, 2002)).

Integrins have a crucial role in metazoan biology by mediating cell-cell adhesion and adhesion to ECM components. Most of the integrins bind ECM components recognizing a particular Arg-Gly-Asp (RGD) motif present in proteins like vitronectin (VN) or fibronectin (FN). Another motif, functionally related to RGD motif and usually found in many integrin ligands, is the LDV-motif (Leu-Asp-Val). Specific motifs are present also in other particular matrix proteins like collagen (COL) or laminin (LN). Many counter-receptors are integrin ligands, reflecting the role of integrins in cell adhesion especially in the blood cells. Moreover, integrins-ECM interaction transmits signals across the plasma membrane regulating cell migration, survival, cell cycle progression and modulating differentiation.

As consequence they are involved in physiological processes such as immunity, inflammation, haemostasis, tissue morphogenesis and development. A subfamily of integrins is expressed exclusively on blood cells, mediating cell-to-cell interactions and allowing processes like leukocyte transmigration and platelet aggregation.

Deregulated integrin function can contribute to many pathological scenarios like autoimmunity and cancer. Additionally, the integrin proficiency in controlling ECM topology and cell polarity during migration, poses a direct link to metastasis dissemination.

The function of each of the 24 integrin types is specific and non-redundant. Indeed, despite partial overlaps in substrate specificity, the phenotypes of knockout mice are distinct, reflecting the various roles of different integrins.

INTEGRIN BIDIRECTIONALITY

Integrin are non-canonical signaling receptor as they transmit signals in two directions with different biological consequences. Indeed, integrin-mediated interaction with extracellular components is translated in intracellular signaling (outside-in signaling) but also intracellular signaling can induce changes in integrin extracellular conformation (inside-out signaling). During inside-out signaling integrin activators bind to the cytoplasmic subunit, triggering conformational changes that increase the affinity for extracellular ligands in a process termed "integrin activation". In fact integrins are expressed on the cell surface in an inactive conformation that cannot efficiently bind extracellular ligands (Ginsberg et al., 1992). This is crucial for the immune system functionality, where integrins have to be inactive in resting cells in order to avoid abnormal interaction with endothelial cells. Another example of the subtle regulation of integrin activation can be found in platelets where integrins, in normal conditions, has to be inactive to prevent aggregates leading to thrombosis. Integrin-independent stimuli through GPCRs, T-cell receptor or selectins induce signaling cascades that initiate the integrin activation process. Interestingly integrin can be activated by the interaction with their cognate ligands, linking inside-out to outside-in signaling (Schwartz et al., 1995; Takagi et al., 2002). Blood cells underline the importance of balanced integrin activation/inactivation, even if not all of the integrin are believed to behave so strictly. The activity of integrin in non-blood cells could be regulated in a more localized way, allowing complex processes like cell migration where cell adhesion has to modulated spatially and temporally (Ridley et al., 2003).

Integrins in high-affinity/active conformation bind tightly the ECM component but individually can provide only a weak adhesive force. The avidity of the Integrin-ECM interaction has to be increased so that thousand of weak adhesive forces sum into an efficient adhesive unit. This process is called “integrin clustering” and leads to the formation of specialized cellular structure termed focal adhesion. Activated and clustered integrins assemble large protein complexes on their short cytoplasmic tails, transmitting a wide variety of intracellular signaling in a process called inside-out signaling. The multivalent properties of many ECM components contribute to integrin clustering and localize integrin signaling into discrete regions of the plasma membrane. Outside-in signaling causes a rapid phosphorylation of specific proteins and the activation of lipid kinases. This first early event is followed by the up-regulation of Rho GTPases activity, which modulates cell contractility, polarization and actin polymerization. Finally integrin signaling can influence gene expression inducing survival, proliferation and modulating the genetic program of cell differentiation.

MECHANISM OF INTEGRIN REGULATION

The shift between high and low affinity conformation rules the integrin adhesive properties. During this process, radical changes in the integrin conformation occurs both in the intra and extra cellular regions. The key event in this process relies on the interaction of cytoplasmic proteins with the integrin tails. These interactions trigger the exposure of the ligand-binding site on the extracellular side (LIBS) and provide connection with the actin cytoskeleton.

Integrin extracellular domain structure and conformational changes

Integrins are non-covalently-bound α/β heterodimers composed by large extracellular domains (approximately 800 amino acids) that contain the ligand binding site, single transmembrane domains (TM approximately 20 amino acids) and short cytoplasmic tails (13 to 70 amino acids). The extracellular part of the α-subunit is formed by an N-terminal seven-bladed β-propeller, an Ig-like Thigh domain, and two Calf domains. The extracellular region of the β-subunit is composed by an N-terminal βI domain connected with a hybrid domain, a PSI (plexin/semaphorin/integrin) domain followed by 4 EGF-like domains and a proximal β tail domain (βTD). The head of the integrin is therefore composed by the β-propeller and βI domain and it is supported by the legs formed by the two integrin subunits (Fig. 2a and Fig. 2b).

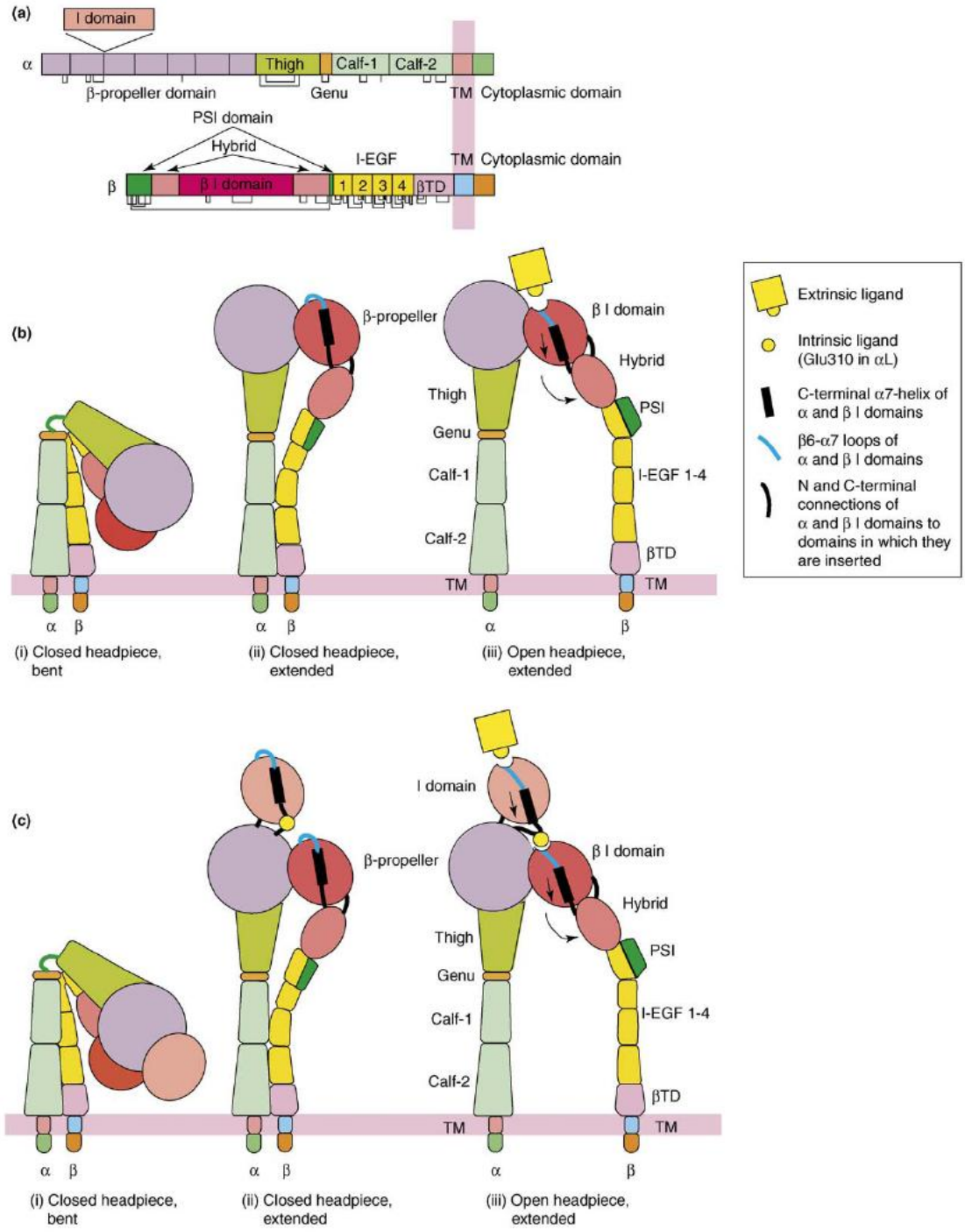


Figure 2 Conformational changes in integrin extracellular domains. **A** Schematic representation of the domains composing integrin extracellular region. I domain that characterizes a particular subset of integrins is indicated. **B** Representation of the conformational changes that lead to integrin open/active conformation in non-I domain integrins or in I domain containing integrins **C** Representation of the conformational changes that lead to integrin open/active conformation in I domain containing integrins . (The schemes represents the principle of switchblade model in which the swing out of hybrid domain requires first integrin extension.) (adapted from (Luo and Springer, 2006))

At least half of the α-subunits contain an additional domain called I domain which constitutes the ligand binding site, thanks to the presence of a metal ion–dependent adhesion site (MIDAS) that coordinates divalent cations (Lee et al., 1995). Interestingly the βI domain is analogous to the I domain of α-subunit and contains a MIDAS as well. In absence of the I domain the ligand binding site is composed by the metal ion occupied MIDAS of βI domain and propeller domain of the α-subunit. Mutations in the MIDAS (Asp119—Tyr in β_3 integrin (Loftus et al., 1990) and Asp130—Ala in β_1 integrin (Takada et al., 1992)) result in complete abrogation of the integrin interaction with their ligands. A crystal structure of $\alpha_v\beta_3$ integrin in complex with cyclic RGD peptide (a motif present in many integrin ligands) showed that Arg contacts the β-propeller of α_v subunit while the Asp interacts with a metal ion (Mn^{2+}) coordinated by the MIDAS (Xiong et al., 2002) . In I domain containing integrins a Glu residue in the I domain interacts, as an intrinsic ligand, with the MIDAS of βI domain coordinating a metal ion (Alonso et al., 2002) (Fig. 2c).The I domain in place constitutes the integrin binding site and contacts integrin ligands through its own MIDAS. Adjacent to the MIDAS there is another metal ion coordinating site (AMIDAS) that can be occupied by a favored Ca^{2+}, stabilizing the inactive conformation.

Integrins can adopt closed and open conformations, which correspond to a different binding capacity. In particular, three major conformational states have been described: “inactive” bent with closed headpiece (with low affinity), “primed” or “active” extended with closed headpiece (with high affinity) and “ligand occupied” extended with open headpiece (Fig.2b). Intuitively in the closed conformation the head of the integrin heterodimer is facing the plasma membrane, being in this way in an unfavorable position to mediate ligand binding. In favor of this hypothesis integrins locked in extreme bent conformation are unable to bind the ligand (Takagi et al., 2002). In addition electron microscopy studies show that integrins adopt a bent conformation when C-termini are clasped or in Ca^{2+} containing buffer. The fact that Ca^{2+} or C-termini clasping abrogate

integrin activation links integrin inactive state to a close conformation (Takagi et al., 2002). In agreement with these studies fluorescent resonance energy transfer (FRET) studies showed that "focal adhesion are sites of integrin extension" (Askari et al., 2010), liking integrin in the extended conformation to the major cell-adhesion sites in cells. Finally specific epitopes, unmasked during the integrin activation process (Lu et al., 2001a), are buried in the bent conformation (Beglova et al., 2002). However, the first crystal structure of $\alpha_v\beta_3$ integrin revealed an unexpected bent conformation (Xiong et al., 2001). This structure has been obtained in Ca^{2+} containing buffer and without a bound ligand, conditions that usually keep integrins inactive. A second $\alpha_v\beta_3$ integrin crystal structure was solved in the presence of Mn^{2+} (metal ion that bind to the MIDAS contributing to integrin activation) and high-affinity RGD peptide. Surprisingly, compared with the previous one, little structural changes were observed (Xiong et al., 2002). Thus, these two crystal structures show that even in potentially activating conditions integrins can adopt a close conformation. Moreover electron microscopy images showed bent $\alpha_v\beta_3$ integrin in complex with FN (Adair et al., 2005).

Based on these evidences, two different theories for integrin activation have been formulated. In both theories, the two key elements are the "swing-out" of the hybrid domain and the extension of the integrin legs. A first theory, called "switchblade model", predicts that integrin will get first in the extended conformation and then interacts with their ligands. A direct consequence of this model is the inability of bent integrin to interact with ligands. According to this model, in order to allow the swing-out of the hybrid domain integrins should get first "fully extended on the knees" (Fig. 2). Indeed also under potentially inactivating conditions, EM images showed integrin molecules to adopt different degrees of bending. This data, if extrapolated to cellular systems, would predict a sort of "breathing movement" of integrins on the cell surface that would place the ligand binding site away from the plasma membrane. In favor of this hypothesis a monoclonal

antibody, (4B4) that prevents hybrid domain swing out, abrogates integrin-ligand interaction stabilizing the low affinity state (Luo et al., 2004b).

A second model, called deadbolt model, predicts that integrins adopt the extended conformation only after ligand binding. In particular, the interaction between βTD (the deadbolt) and βI domain keeps integrin in closed conformation. When this interaction is released, integrins will turn into the active state. In a second step, ligand binding provides the energy for hybrid domain swing-out corresponding to integrin extension. This model could explain the documented capability of integrins to interact with their ligands in the bent conformation, as well as the data in the crystal structures. Moreover this theory takes into account the role played by traction forces in integrin extension and activation (Friedland et al., 2009). However mutations in the βTD domain failed to activate integrins (Zhu et al., 2007).

Additional studies are required in order to fully understand the conformational changes taking places during integrin activation.

Role of transmembrane domains and integrin tails

Integrin TM domain and cytoplasmic tails play a key role in the integrin activation process. The only crystallographic data providing insight into the structure of TM domains derives from $\alpha_{IIb}\beta_3$ integrin. α_{IIb} TM domain is as short straight α-helix (24 residues) followed by a backbone reversal that packs Phe992-Phe993. β_3 TM is a linear α-helix of 30 residues. Membrane embedding studies predicts that β_3 TM is longer than the width of a lipid bi-layer, which imply a 25° tilt in order to maintain membrane embedding (Lau et al., 2008) (Fig.3b and Fig. 3a).

The association of the TM and cytoplasmic domains controls integrin bidirectional signaling. In particular the TM domains interaction maintains the inactive state, while the perturbation of this interaction leads to integrin activation (Hughes et al., 1996). Interestingly mutations that shorten the TM domains length or destroy the TM surface of interaction are uniformly activating (Hughes et al., 1996) (Partridge et al., 2005) while the introduction of disulfide bridges prevents TM separation and abolishes integrin activation (Luo et al., 2004a). In particular cysteine-scanning experiments show that disulphide bonded integrins cannot bind their ligands even in the presence of other activating mutations. These evidences suggest that a complete TM domain separation is needed for integrin activation and supports the notion that TM separation corresponds to active state. Interestingly disulphide-bonded integrins can be activated, in an outside-in fashion, by antibody and Mn^{2+}, implying that outside-in signaling does not require TM domain separation (Luo et al., 2004a).

Crystal structures reveal that TM domain dimerization is supported by two distinct elements termed inner membrane clasp (IMC) and outer membrane clasp (OMC) (Lau et al., 2009). The OMC forms thanks to the packaging interaction between canonical coiled-coil dimerization motifs (GxxxG) from both TM domains (Fig 3c). In this way Arg995 of α_{IIb} interacts with Asp723 of β_3 allowing the formation of a salt bridge that stabilizes TM domain interaction. The Asp-Arg salt bridge constitutes the IMC (Fig. 3d). Mutational analysis showed that disruption of salt bridge in the IMC triggers β_3 integrin activation (Hughes et al., 1996) and subsequent studies validated the same principle also in β_1 and β_2 integrin (Imai et al., 2008). However, knock-in mice in which β_1 integrin IMC was disrupted did not display any significant phenotype (Czuchra et al., 2006).

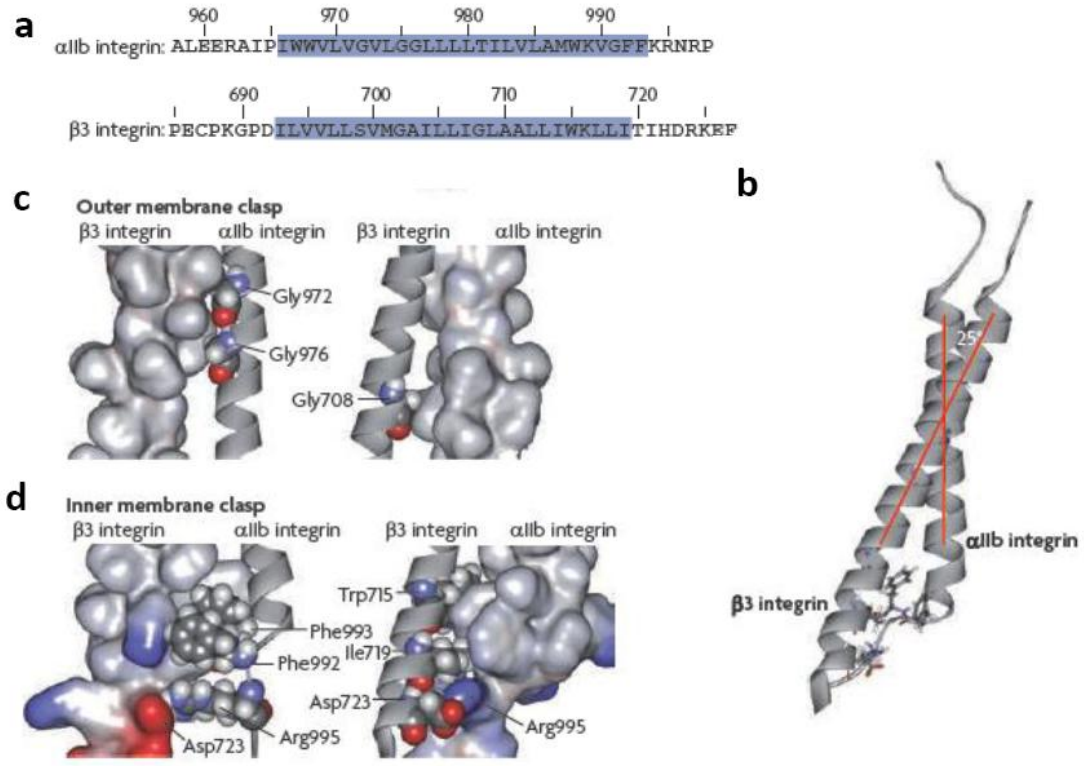


Figure 3 Structure of integrins TM domain. **A** sequence of α_{IIb} and β_3 TM domains. The membrane embedded residues are highlighted in blue. **B** Structures of integrin TM domains with the characteristic crossing-angle of 25°. The side chains represented are the one of the residues involved in the formation of the salt bridge (Arg995 of α_{IIb} and Asp723 of β_3). **C** representation of the outer membrane clasp and the molecular interaction that supports it (left: front view, right: back view) **D** representation of the inner membrane clasp and the molecular interaction that supports it (left: front view, right back view)adapted from (Shattil et al., 2010))

Different mechanism can be accounted for integrin TM domain separation. The amount of β integrin TM residues could be shortened upon activation, allowing the straightening of the TM domain. In the piston model, piston-like movement of TM domains could destroy the dimerization interface, causing TM separation. Alternatively, in the scissor model the small crossing angle of the TM domains during resting state increases to a large crossing angle in the activated state.

Integrin tails are rather short and posses no enzymatic activity. Together with TM domains, integrin tails undergo complete separation during activation. This is supported by numerous studies showing how the clasping or the constitutively disulphide liking of cytoplasmic domains inhibit integrin activation (Lu et al., 2001b) (Luo et al., 2004a). In another elegant study, FRET has been measured between integrin subunits fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) in the cytoplasmic regions (Kim et al., 2003). Fluorescent energy transfer occurred only in integrins in resting state, demonstrating the cytoplasmic domains to be close together in the inactive conformation. Consistently, upon inside-out signaling, FRET was greatly reduced demonstrating that, together with TM domains separation, cytoplasmic tails separate by more than 100 Å. On the other hand outside-in activation by Mn^{2+} did not reduced FRET, although subsequent ligand engagement did. However, additional studies on purified integrin tails indicate that their interaction is extremely weak and for this reason in many studies was no detected.

Integrin cytoplasmic tails, and especially the β subunit tail, are the core of integrin regulation. Despite their short length, many proteins have been shown to bind directly to three conserved "hot-spot". The increasing number of direct interactors, together with the limited number of binding sites in the integrin tails, suggest significant overlaps and competitions between the adaptor binding sites (Fig 4).

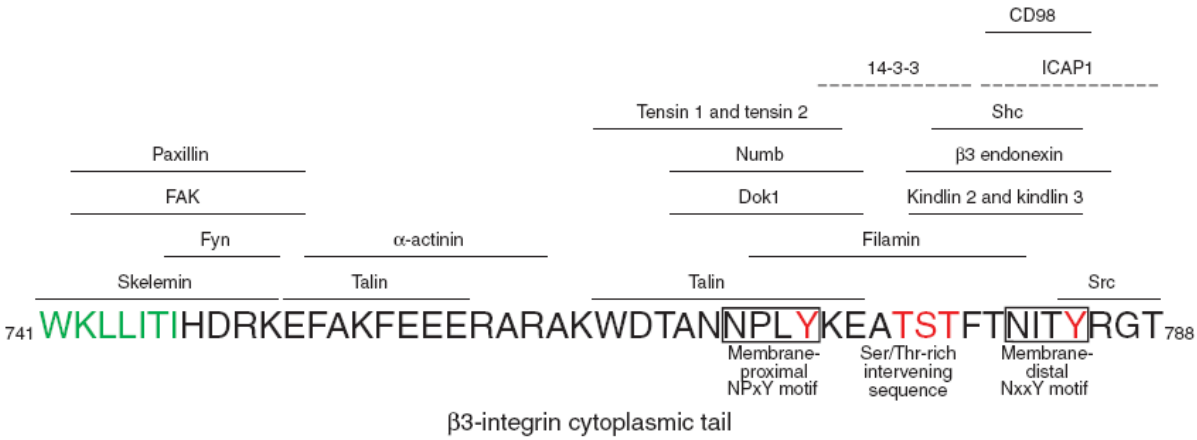


Figure 4 Aminoacid sequence and adaptor-sites of β_3 integrin cytoplasmic domain. The membrane proximal and distal NxxY motifs and the ser/thr rich regions are indicated. Residues that can be phosphorylated are indicated in red while residues buried in the membrane are indicated in green. The several proteins that interact with integrin tail are indicated by solid lines in correspondence of their binding sites. Broken lines represent adaptors that bind to other β subunits. (adapted from (Legate and Fassler, 2009))

The first adaptor-binding site is a membrane-proximal HDRK motif that is involved in the formation of the salt bridge that keeps integrin TM and cytoplasmic domains close together in the inactive state. This motif is unmasked upon tails separation occurring during integrin activation. HDRK motif has been shown to be a binding site for paxillin (Schaller et al., 1995), skelemin (Reddy et al., 1998), Src family kinase Fyn (Reddy et al., 2008) and FAK, although the integrin-FAK interaction has been shown to occur through paxillin (Hayashi et al., 2002).

The other two hot-spot in β subunit tail are two well conserved NxxY motifs characterized by a canonical recognition sequence for phosphotyrosine-binding (PTB) domains. In particular integrin tails contains a membrane-proximal NPxY motif and a membrane-distal NxxY motif. Proteins containing PTB domains like Numb, Dok1, ICAP1 and tensin have been shown to directly interact with different integrin β tails (Calderwood et al., 2003) (Fig. 4).

Other two proteins (talin and kindlin) bind directly the NxxY motifs of β integrin tails and control the integrin activation process: in particular, talin interacts with membrane-proximal NPxY motif whereas kindlin interacts with the membrane-distal NxxY motif. Interestingly both of them contain a PTB domain that falls into a larger band (4.1, ezrin, radixin, moesin) termed FERM domain (see below). In general, proteins that bind to the membrane-proximal NPxY motif can interact with cytoplasmic tails of different integrin subunits while proteins that bind to the membrane-distal NxxY motif interact only with some types of integrin. One possible reason for this is the sequence divergence within NxxY motif respect to the membrane-proximal motif (Fig.5).

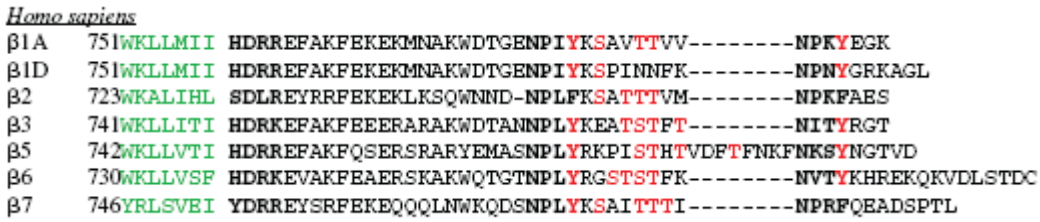


Figure 5 Alignment of the different β subunits sequences in humans. Residues buried in the membrane that become available upon integrin activation are indicated in green. NxxY and HDRK motifs are indicated in bold. Residues that can be phosphorylated are indicated in red. (adapted from (Legate and Fassler, 2009))

For example, ICAP1 binds only the membrane-distal NPKY motif of β_1 integrin. ICAP1 competes with talin and negatively regulates integrin activation (Chang et al., 2002). β_3 endonexin does not contain a PTB domain and interact only with membrane-distal NxxY motif of β_3 integrin (Eigenthaler et al., 1997). Another direct interactor is filamin that binds between membrane-proximal and distal motifs. Filamin interaction sterically inhibits talin binding and has dramatic effects on integrin activation (Kiema et al., 2006).

The tyrosines within NxxY motifs of β_1 and β_3 integrin motifs can be phosphorylated by SRC family kinases (Sakai et al., 2001) (Law et al., 1996). Interestingly, despite the misleading nomenclature, PTB domains binds preferentially to non-phosphorylated tyrosine in NxxY motifs and, in some cases, phosphorylation inhibits their interaction. Moreover the importance of integrin phosphorylation seems to be strictly integrin-specific. In fact knock-in mice carrying a double phenylalanine substitution in the two NxxY motifs of β_1 integrin displayed no visible phenotype (Czuchra et al., 2006) while the same mutations in β_3 integrin causes to bleeding (Law et al., 1999).

In addition, also the serines and the threonines between the two NxxY motifs can be phosphorylated, being the third major site of phosphorylation in integrin tails. Phosphorylation is thought to be a regulator of the interaction of PTB containing proteins with the integrin tails. Upon phosphorylation, PTB domains with high affinity for the unphopshorylated forms will be displaced favoring the interaction with the "phospho-affine" ones. A typical example of this principle is Dok1, whose affinity for β_3 integrin membrane-proximal NPxY motif is increased upon phosphorylation. Moreover NPxY motif phosphorylation inhibits talin interaction, allowing Dok1 to compete with talin and to negatively regulate integrin activation (Oxley et al., 2008).

A phosphorylation switch has been observed also for filamin and talin. Filamin binds to unphosphorylated serin/threonine region between the two NxxY motifs, competing with talin binding to β_7 integrin tails. Threonine phosphorylation impairs filamin binding but does not influence talin interaction. In this way talin is free to bind β_7 integrin tails, regulating integrin activity (Kiema et al., 2006).

Talin

Talin is a ~270 kDa protein composed by a globular N-terminal head domain (47 kDa) and a long C-terminal rod domain. There are two isoforms of this protein: talin1 and talin2. The head domain contains a FERM domain composed by 3 subunits (F1, F2 and F3) and an extra F0 domain. The F3 subunit contains a PTB domain that mediates the direct interaction with β integrin tails in membrane-proximal NPxY motif. In addition, talin head domain interacts with hyaluronan receptor, a spliced variant of phosphadylinositol (4)-phosphate5-kinase type Iγ, focal adhesion kinase (FAK) and contains a filamentous actin (F-actin) binding site. The rod domain is a large flexible structure formed by helical bundles that contains an additional integrin binding site, two F-actin binding sites and several vinculin binding sites. The end of rod domain contains a dimerization sequence (Critchley and Gingras, 2008). The linker region between head a rod domain can be cleaved by protease calpain 2.

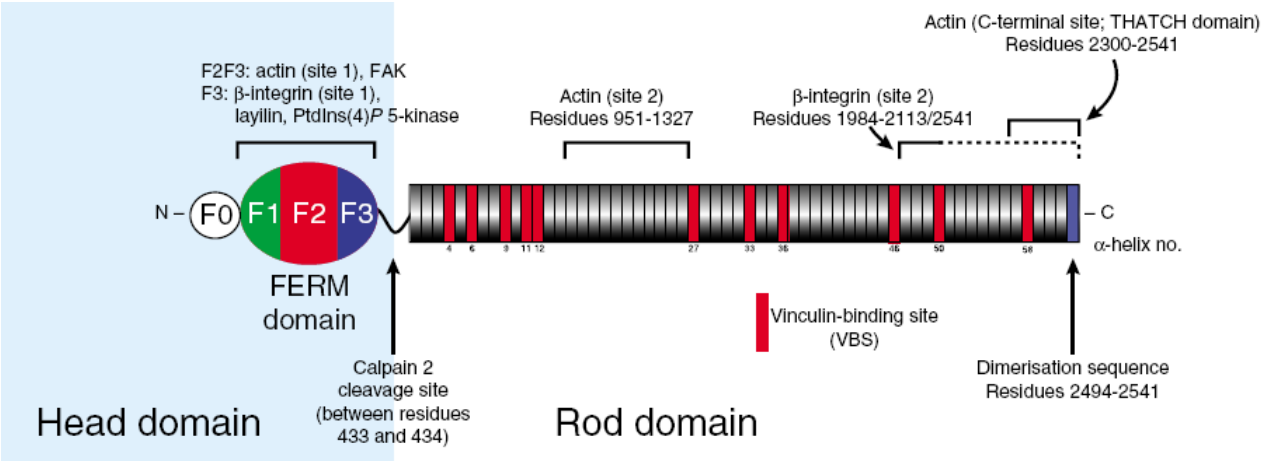


Figure 6 Structure of talin. Talin head domain is divided in the 4 subunits (F0, F1, F2, and F3). In talin Rod domain the interaction sites for actin, vinculin and integrins are indicated (adapted from (Critchley and Gingras, 2008)).

Talin was at first identified as an interactor of the cell-substrate attachment (CSAT) antigen (later on identified as an integrin). Talin-CSAT interaction mediates the connection between ECM and actin cytoskeleton in adhesion plaques (Horwitz et al., 1986). The first evidence of its role in integrin activation are found in studies in CHO cells, where talin expression increases the affinity of normally inactive integrins (Calderwood et al., 1999). Moreover, this study shows that talin head domain is sufficient to activate integrins. An elegant mutational analysis, together with knock-down experiments, identified talin-integrin interaction as a final common step in integrin activation. In particular mutations in talin PTB domain and in integrin NPxY motif impair talin binding and abolish integrin activation, giving rise to adhesive-deficient phenotypes (Tadokoro et al., 2003).

Interestingly talin is not the only PTB domain containing protein that can interact with β integrin tails. Indeed several other molecules posses a PTB domain and bind to

membrane-prossimal or –distal NxxY motifs (see above). However, only talin specifically activates integrin whereas other PTB proteins do not. Structural and crystallographic analysis clarify this point demonstrating that talin, beside binding the membrane-proximal NPxY motif, interacts also with another membrane-proximal region where two phenylalanine residues play a crucial role (Wegener et al., 2007). Interestingly other NPxY binding proteins, like Dok1, display only the first interactions with the membrane proximal NPxY motif. Thus, it is proposed that talin F3 subunit interacts at first with the NPxY motif. Afterwards an extra loop, located in F3 subunit, interacts with another membrane-proximal region containing Phe residues. Interestingly mutation of the key residues for the second "talin-specific" interaction abolished integrin activation without impairing talin binding (Vinogradova et al., 2002).

NMR and FRET studies further unveiled the mechanism behind integrin activation by talin. Talin has been shown to compete with α integrin tail for the binding to the β integrin tails. Talin binding to β integrin tail destabilizes the interaction that keeps TM and cytoplasmic domain close together during the inactive state (Vinogradova et al., 2000). In fact, talin-integrin interaction impairs FRET between the integrin tails, indicating a separation of the cytoplasmic domains (Kim et al., 2003). A detailed description of the changes occurring in integrin TM and cytoplasmic domains upon talin interaction derives from structure-function analysis. Talin F3- β integrin interaction stabilizes the helical structure of β integrin tail and orients a group of Lys residues toward the negatively charged phospholipids of the plasma membrane (Wegener et al., 2007). A second structural study (Anthis et al., 2009) indicates that talin can electrostatically interact with the Asp residue responsible for the salt bridge (Asp 723 of β_3 integrin with Arg 995 of α_{IIb} integrin) that stabilizes the transmembrane complex during inactive state. Consequently, talin could disrupt the electrostatic interaction between α and β subunits, triggering integrin activation. This study identified also an additional basic patch in F2

subunit that, by interacting with the plasmamembrane, alters the angle of β TM domain (Fig. 7). The capacity of talin in inducing structural changes, altering the tilt angle of β integrin tail, explains why integrin activating mutations, that simply impair cytoplasmic tail interactions, fails in activating integrin in absence of talin (Tadokoro et al., 2003).

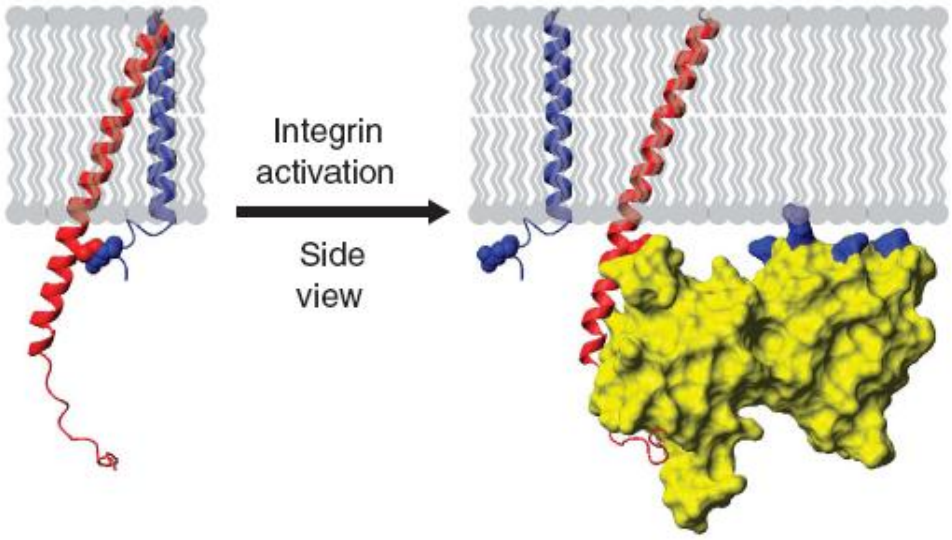


Figure 7 Mechanism of integrin activation by talin. Integrin in inactive state with cytoplasmic domains interactions and tilt of 25° is represented on the left. On the right the active state, where talin cause integrin tails separation by binding β integrin cytoplasmic domain and interacting with the inner side of the plasma membrane, is represented (adapted from (Anthis et al., 2009))

Integrin activation has to be strictly controlled during cellular processes, implying a tight regulation of integrin-talin interaction. The investigation on the mechanisms of talin activation, targeting to focal adhesion and interaction with integrin tails start to shed light on this process.

A NMR analysis showed that talin carries an auto-inhibitory interaction between the PTB domain and the C-terminal part of the rod domain (Goksoy et al., 2008). This study raises the possibility that when integrin has to assume an inactive conformation, talin function is auto-inhibited. Moreover the interaction with phosphatidylinsotitol-4,5-

bisphospshate (PIP2) has been shown to disrupt the auto-inhibitory interface in talin, enhancing talin-integrin interaction (Goksoy et al., 2008).

Stimulation of cells with agonists triggers integrin activation and the signaling pathways responsible for this process have been recently clarified. RAP1 and RAP2 are two Ras subfamily members that induce integrin activation. Being small GTPases, they cycle between an active GTP-bound and an inactive GDP-bound form. RAPs function is dependent on the interaction with its effector, RAP1-GTP-interacting adaptor molecule (RIAM, a member of a family of adaptor proteins that includes also lamellipodin). RIAM expression is enriched in hematopoietic cells whereas lamellipodin is a parologue expressed in fibroblasts (Boettner and Van Aelst, 2009). RIAM overexpression has been shown to stimulate while its knock-down to block talin recruitment to integrins tails in living cells (Watanabe et al., 2008).

A further analysis showed that RIAM scaffold function connects the membrane targeting sequences of RAP1 to talin, targeting talin to the plasma membrane and allowing its interaction with integrins (Lee et al., 2009). A third mechanism of regulation is integrin tail phosphorylation (see above). A structural analysis showed that integrin-talin interaction (between PTB domain and NPxY motif) is supported by acidic and hydrophobic interactions that are abolished by the introduction of a phosphate group (Garcia-Alvarez et al., 2003).

Kindlin

Kindlins are a family of evolutionary conserved multidomain proteins whose mutation causes the Kindler syndrome (Siegel et al., 2003). Three kindlins family members have been discovered: kindlin-1 (URP) expressed predominantly in epithelial cells, kindlin-2 (Mig2) expressed in all tissues with a particular enrichment in skeletal and smooth muscle cells and kindlin-3 (URP3) expressed only in hematopoietic cells. In particular Kindler syndrome, which names kindlin proteins, is a blistering disease characterized by an epithelial cell-adhesion defect (Siegel et al., 2003). Kindlin-3 mutation causes a rare Leukocyte-adhesion deficiency (LAD) type III that results in impaired leukocyte and platelet cell-matrix interaction (Kuijpers et al., 2009).

Kindlin, unlike most of FERM domain containing proteins, localized its FERM domain at the C-terminal part. Interestingly kindlin FERM domain is split in the F2 subdomain by a pleckstrin homology (PH) domain (Fig.7). Structural analysis shows that kindlin FERM domain and PTB domain in F3 have high sequence and structural similarity with the talin ones.

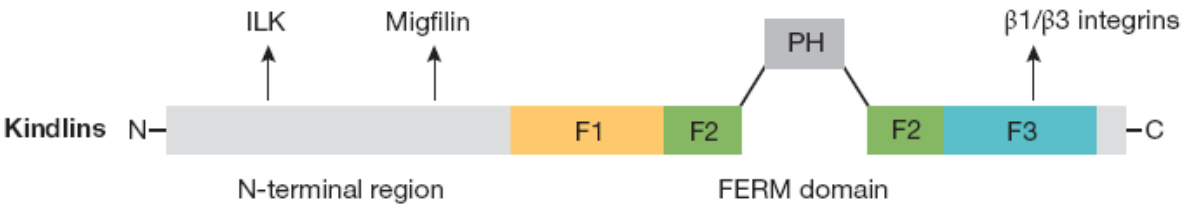


Figure 7: Schematic representation of kindlin. The FERM domain is composed by F1, F2 and F3 subunits. F2 subunit is interrupted by a PH domain. Interaction sites for integrins, ILK and migfilin are indicated (adapted from (Larjava et al., 2008)).

The analysis of kindlin-linked pathologies together with *in vivo* and *in vitro* studies confirmed the essential regulatory function that kindlin exerts on integrin activation. Genetic depletion of kindlin-1 in mice results in a skin phenotype reminiscent of Kindler syndrome (Ussar et al., 2008). Kindlin-2 deficiency impairs integrin function in epiblast and endoderm cells resulting in defective implantation. This phenotype is consistent with ubiquitous expression of this protein (Montanez et al., 2008). The lack of kinlin-3 abolishes $\alpha_{IIb}\beta_3$ integrin interaction with its ligands and causes a defective platelet aggregation (Moser et al., 2008) whereas leukocytes lacking kindlin-3 cannot transmigrate through wall vessel (Moser et al., 2009a).

The function of kindlin in regulating integrin activation was directly observed for the first time in a study in which kindlin was shown to interact and activate $\alpha_{IIb}\beta_3$ integrin in CHO cells (Shi et al., 2007). The following biochemical experiments confirmed the direct interaction between kindlin and β_1, β_2 and β_3 integrin tails (Montanez et al., 2008) (Moser et al., 2008) (Moser et al., 2009a) (Ma et al., 2008). In particular kindlin was shown to bind the membrane-distal NxxY motif in β integrin tails through its PTB domain in F3. An additional kindlin interaction-site has been localized in a short Ser/Thr rich region that lies between membrane-proximal and distal NxxY motifs (Harburger et al., 2009) (Montanez et al., 2008). Interestingly mutations in talin binding site do not abrogates

kindlin binding as mutations in kindlin binding sites do not impair talin recruitment (Montanez et al., 2008). However, mutations in talin or kindlin specific NxxY motif as well as mutations in their PTB domains block integrin activation, indicating a sort of cooperation of these molecules in the integrin activation process. Indeed studies in CHO cells showed that co-expression of kindlin-2 and talin head domain synergistically enhanced integrin activation while knock-down of kindlin-2 had a crucial negative effect (Ma et al., 2008). However the stimulatory effect of kindlin was lost in absence of talin (Harburger et al., 2009) demonstrating that the amount of talin in the cells determine kindlin efficacy.

Interestingly some observations support the notion that kindlin posses also a integrin-inhibitory effect related to its level of expression. Indeed over-expression of kindlin-1 and 2 represses integrin activation independently of integrin-kindlin interaction. Moreover kindlin-1 and 2 activate $\alpha_{IIb}\beta_3$ integrin when co-expressed with talin head domain but cannot cooperate with talin in $\alpha_5\beta_1$ integrin activation, showing an unexpected inhibitory effect on talin function (Harburger et al., 2009). The integrin-independent effect of kindlin is demonstrated by the analysis of a cell type that does not express any integrins. Indeed kindlin deficient erythrocytes showed structural membranes defects (Kruger et al., 2008).

Besides the binding with integrins, kindlin also interacts with integrin linked kinase (ILK) and migfilin, a filamin binding protein (Tu et al., 2003). Indeed both ILK and migfilin co-localization in focal adhesions depends on kindlin. Moreover this interaction allows kindlin to establish an indirect connection with the actin cytoskeleton and participate to signal transduction.

The mechanisms of talin-kindlin cooperation in integrin activation are still unclear and putative cross-talk models have been speculated. In a first scenario kindlin-integrin interaction could facilitate talin interaction with consequent displacement of talin.

However, since talin and kindlin binding sites are distinct, they could simultaneously interact with the same integrin tail, although the order of the interaction is still unclear. Alternatively, talin and kindlin could interact with distinct integrin tails, cooperating in trans to integrin activation.

Integrin clustering and focal adhesion assembly:

Integrins in the active state bind a wide range of ligands in the ECM. However, in order to mediate strong cell-adhesion, individual adhesive units have to assemble into a larger adhesive platform. This phenomenon is termed "integrin clustering" and is defined as the association of single heterodimers to form heterooligomers. In this way integrins cluster in transient early structures called "nascent adhesions" (Choi et al., 2008). Their molecular composition is not known but likely they contain the primitive integrin activation complex composed by talin (and eventually kindlin) providing a first connection to the actin cytoskeleton. Nascent adhesions can progress to the stage of dot-like focal complexes, that appears like spots of 100 nm and are composed by several hundred molecules (Geiger et al., 2001). Focal complexes occurs usually underneath the lamellipodia (Alexandrova et al., 2008), flat and pro-migratory cellular structures generated by actin polymerization. Focal complex assembly is dependent on forces acting on adhesion sites that derives from the periodic uplifting of the lamellipodia and myosin II-mediated contractility (Giannone et al., 2007). Focal complexes can mature into larger "focal adhesions" enriched in α-actinin (FAs 3-10 μm) and finally in fibrillar adhesions (Geiger et al., 2001). Podosome and invadosome are the counter-part of FAs in osteoclast/macrophages and in cancer cells respectively. Generally, focal complexes are a feature of migrating cells whereas mature

FAs are found in resting cells. The transition from focal complex to FAs occurs in the boundary between lamellipodia and lamella (a flat structure in cell periphery but more internal respect to lamellipoida) and requires α-actinin and myosin II. Interestingly the motor activity of myosin II seems to be dispensable for this transition. However the capability of myosin II in promoting actin stress fibers is required for a complete FAs maturation (Choi et al., 2008).

Many factors contribute to integrin clustering. Inside-out signaling stimulates the recruitment of multivalent proteins on the integrin cytoplasmic domains, inducing integrin clustering. The intact talin molecule is required for focal adhesion assembly whereas talin head, beside the integrin stimulatory effect, is not sufficient for FAs formation (Zhang et al., 2008). Moreover, talin dimerization is required for its localization into focal adhesion and presumably for its clustering activity (Smith and McCann, 2007). Talin posseses two integrin binding sites (one in head F3 domain and one in the rod domain) meaning that a talin-dimer binds up to four integrin tails simultaneously. Thus, talin could have an intrinsic integrin clustering activity that would explain why calpain cleavage, by dissociating talin head from the rod domain, induces focal adhesion disassembly (Franco et al., 2004). Several evidences demonstrate that integrin clustering requires an active conformation of the receptors, PIP2 and immobilized ligands (Cluzel et al., 2005). In addition PIP2 binding to talin in F2 and F3 domains is required for its clustering activity (Saltel et al., 2009). A novel role of kindlin in focal adhesion assembly has been suggested by the reduced number of FAs in cell lacking kindlin-2, even in the presence of integrin activating stimuli (Montanez et al., 2008). Integrin clustering is induced also by the binding of multivalent ligand in the ECM and is enhanced by the release of integrin from cytoskeletal constrains(Buensuceso et al., 2003).

OUTSIDE-IN (FOCAL ADHESION) SIGNALING

Activated and clustered integrins transmit signaling(s) across the plasma membrane in a process called outside-in signaling. Integrin outside-in signaling usually requires ligand binding (although an increasing number of evidences suggest that integrin activation induce a signaling in a ligand-independent fashion. This is the main topic treated in this PhD thesis and will be analyzed in the discussion). Importantly integrin tails do not display any enzymatic activity and, in order to signal, they are dependent on the binding of effector molecules. The sequential binding of focal adhesion proteins changes focal adhesion composition during their maturation. This process enables integrins to transmit different kinds of signalings in response to different extracellular contexts. Integrin tails and focal adhesion proteins can undergo extensive phosphorylation, creating novel binding sites for other proteins and regulating their signaling activity. Moreover, the incorporation of phosphoinositides into FAs regulates the recruitment of specific proteins. In addition FAs are specialized mechano-transduction platforms. Indeed, they can react to external or internal mechanical forces, modifying their composition and unmasking novel binding sites or phosphorylation motifs. Several studies indicate talin, ILK, vinculin and α-actinin as the major mediators of integrin linkage to the actin cytoskeleton. FAK, paxillin and SRC play a role in the modulation of intracellular signaling through their enzymatic and scaffold activity.

Integrin-cytoskeleton linkage and focal adhesion maturation

The initial integrin-cytoskeleton interaction is provided by the recruitment of talin in focal adhesions. Indeed fibroblasts lacking both talin1 and 2, besides defects in integrin activation, fail to connect integrin to the actin cytoskeleton (Zhang et al., 2008). The role of talin has been investigated also in relation to migration and signaling. Concomitant knock-down of the two talin isoforms (talin 1 and talin 2) severely affects cell spreading, signaling to focal adhesion kinase and traction force generation. The expression of the talin head domain partially rescued these defects by increasing cell-adhesion but it did not reconstitute cytoskeleton linkage. Intact talin molecule was needed to restore cell spreading, focal adhesion assembly and signaling, demonstrating the importance of the interactions with the actin cytoskeleton (Zhang et al., 2008). Moreover, filamin competes with talin for the interaction with integrin tails. Increased filamin binding has been shown to negatively regulate cell migration, whereas talin binding has a stimulatory effect (Calderwood et al., 2001). The crucial role of talin is underlined by the phenotypes of knock-out mice. Indeed talin-1 depletion results in death at gastrulation due to impaired cytoskeletal organization and cell migration (Monkley et al., 2000).

The exact role of kindlin in FAs signaling and maturation is still unclear. However kindlin-3 depleted platelets have reduced cell spreading and abnormal cytoskeletal organization (Moser et al., 2008). Kindlin-2 deficient cells, in which integrins are activated by Mn^{2+}, displayed a serious impairment in FAs assembly and failed to localize ILK in FAs (Montanez et al., 2008). ILK, in turn, can mediate additional connections with the cytoskeleton by interacting with parvin (Tu et al., 2003), reinforcing in this way the integrin-actin linkage. ILK deficient cells display impaired FAs formation, reduced cell

spreading and disorganized actin cytoskeleton (Sakai et al., 2003). Additionally recent evidences indicate a role for ILK in the integrin activation process, as ILK knock-down impairs integrin activation in a talin dependent fashion (Honda et al., 2009).

Once talin interacts with integrin tails, vinculin is rapidly recruited to early nascent adhesions. Vinculin interacts with talin thanks to several binding sites (up to 11 (Gingras et al., 2005) in the talin rod. Interestingly many vinculin-binding sites are inaccessible, being buried in the rod domain. Mechanical stretch of talin rod domain activates vinculin binding by unmasking cryptic binding sites (del Rio et al., 2009). These findings demonstrate how mechanical forces can change the conformation of a FAs protein like talin, suggesting a general mechano-transduction pathway that can translate mechanical forces into intracellular responses. Vinculin depleted fibroblast displays unstable FAs, accelerated FAs turnover and cell migration, demonstrating its crucial role in FAs maturation and assembly (Saunders et al., 2006). Although talin can establish a first link with the cytoskeleton, the absence of vinculin abrogates FAs maturation. Thus, vinculin simultaneous interaction with actin and talin could reinforce cytoskeleton linkage leading to FAs maturation (Humphries et al., 2007). However, vinculin head, which interacts only with talin, has been shown to be sufficient for FAs maturation, indicating other mechanisms of regulation probably involving integrin clustering (Humphries et al., 2007). Another protein shown to interact with both talin and vinculin is α-actinin. α-actinin has been shown to modulate the stability of the cytoskeleton linkage thanks to its actin-bundle activity. Moreover α-actinin is necessary for the transition from focal complexes to focal adhesions through its actin cross-linking activity (Choi et al., 2008). In particular, α-actinin affinity for actin is modulated by FAK phosphorylation (Izaguirre et al., 2001).

Other proteins that contribute to integrin actin-linkage are paxillin and tensin. Paxillin is recruited to early nascent adhesion where, thought direct interactions with FAK, vinculin and SRC, modulates integrin-cytoskeleton linkage and focal adhesion

composition (Laukaitis et al., 2001). Additionally paxillin interacts with α integrin tail and stabilizes integrin-talin interaction (Alon et al., 2005). Tensin is recruited in mature FAs and it is a marker of fibrillar adhesion. Tensin contains an actin-binding domain and can mediate integrin-cytoskeleton interaction. Moreover tensin binds to the NPxY motif exploited by talin. Phosphorylation of the NPxY motif, that occurs during FAs maturation, has been shown to disrupt talin binding in favor of tensin interaction (McCleverty et al., 2007), unveiling a novel phosphorylation-dependent mechanism of regulation. The molecular composition of mature FAs is represented in Fig. 8.

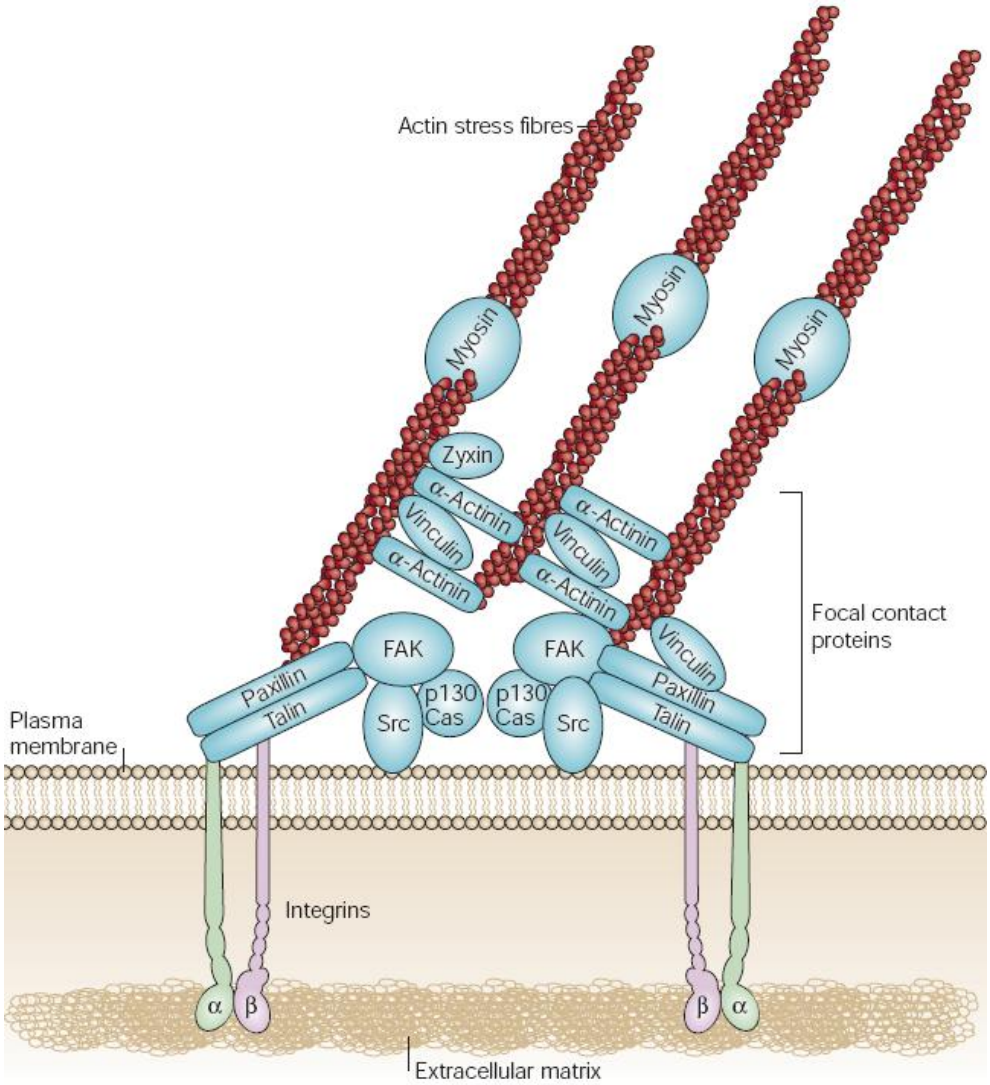


Figure 8 Molecular architecture of FAs. The schematic composition of FAs and the linkage to actin cytoskeleton is represented (adapted from (Mitra et al., 2005)).

Several studies analyzed the diffusion of integrin and FAs components within the plasma membrane (Hu et al., 2007). Integrins engaging the ECM have a low velocity, together with FAs proteins associated with them. Proteins with a weak interaction with integrins but a strong connection with the actin cytoskeleton move faster, slightly behind the actin treadmiling. Talin and vinculin can be associated both with slow-moving integrins or with faster-moving actin. This plasticity allows the transmission of pulling force to the integrin complexes stably associated with actin (crucial for FAs maturation). At the same time weakly actin-associated integrins allows the cell body to slide over the adhesion sites.

Integrin-mediated adhesion in the control of actin dynamics:

Integrin-ECM engagement and the following link to actin cytoskeleton spatially and temporally control the actin cytoskeleton rearrangements. In this way integrin linkage to actin cytoskeleton allows both the control of local actin polymerization and at the same time the global modulation of cytoskeleton dynamics.

Integrins can modulate cell protrusion by assembling a complete actin polymerization machinery on their cytoplasmic domains. A major role is played by the Arp2/3 complex nucleation activity that controls the assembly of actin filaments in a branched fashion during lamellipodia protrusion. The Arp2/3 complex has to be activated by Wiskott-Aldrich Syndrome protein (WASP)/Scar family of activator proteins (Pollard, 2007). Arp2/3 complex is targeted to nascent adhesions by its interaction with FAK and vinculin. Indeed a missing FAK-Arp2/3 complex interaction impairs lamellipodia

protrusion and consequent cell spreading (Serrels et al., 2007). Arp2/3-vinculin interaction is transient and mainly involved in nascent adhesion targeting (DeMali et al., 2002).

Actin cytoskeleton rearrangements are in control of Rho GTPases, a protein family that in mammals is composed by 20 members. Rho GTPases shift between an active GTP-bound form and an inactive GDP-bound form. This cycle is regulated by three specific factors: inactive Rho GTPases are sequestered by Rho-GDP dissociation inhibitors (Rho-GDI), which keep them in the cytoplasm preventing membrane targeting. The release of Rho-GTPases from Rho-GDIs allows membrane localization and subsequent activation by guanine nucleotide exchange factors (GEF) that promote GTP loading. Finally Rho GTPases can return to the inactive states under the activity of GTPase-activating proteins (GAP) that promote GTP hydrolysis (Fig. 9). There are three major Rho GTPases responsible for the control of actin dynamics: RhoA, Rac and Cdc42.

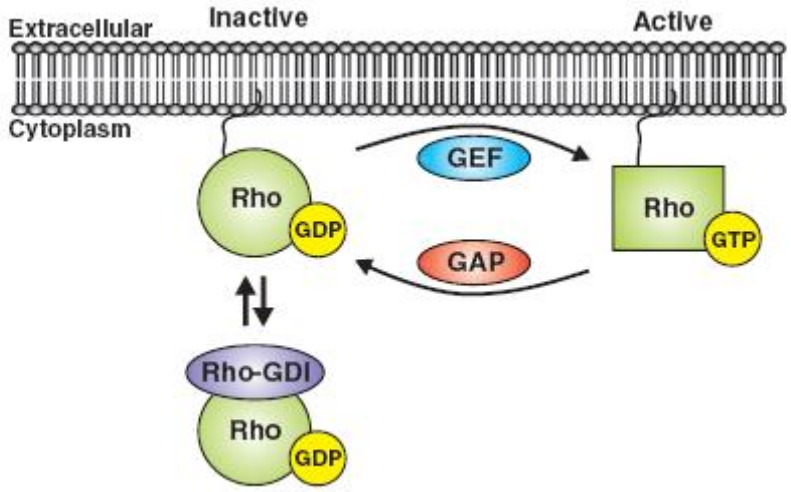


Figure 9 Mechanisms of Rho GTPases regulation. Rho-GDIs sequester inactive/GDP bound Rho in the cytoplasm. The release from Rho-GDIs allows membrane targeting, where GEFs can promote GTP loading corresponding to the active state. GAPs promote GTP hydrolysis that leads to Rho-GDP bound/inactive state(adapted from (Huveneers and Danen, 2009)).

Rac is the main regulator of lamellipodia protrusion. Integrin engagement promotes Rac membrane targeting and activation by the recruitment of numerous GEFs (del Pozo et

al., 2004). In this way, nascent adhesions and focal complexes at the leading edge will be enriched in active Rac that in turn promotes lamellipodia formation. Rac activates the Arp2/3 complex by acting on WAVE/WASP family of Arp2/3 complex activators (Cory and Ridley, 2002). Arp2/3 complex binds to the sides or tips of pre-existing actin filaments and promotes the formation of a second daughter filament in a branched fashion. WAVE/WASP proteins can in turn associate with GAPs and GEFs, creating positive or negative feedback loops that regulates the extent of Rac1 activity (Soderling et al., 2002). Activated Rac can also induce the recruitment and clustering of newly activated integrins to the leading edge (Kiosses et al., 2001), creating a second positive loop.

The role of integrins in activating Rac is underlined by conditional knock-out studies that show decreased level of RAC1 activity in β_1 integrin deficient cells (Nodari et al., 2007).

Cdc42 is the master regulator of cell polarity. Cdc42 activity is located at the leading edge of cells and its inhibition or delocalized activation abolish directional migration (Etienne-Manneville and Hall, 2002). Integrin-matrix interaction is required for Cdc42-mediated cell polarization, as RGD peptides, by blocking integrin engagement, impair Cdc42 activation and cell polarization (Etienne-Manneville and Hall, 2001). Cdc42 influences cell polarity by restricting the sites of lamellipodia formation through the action of Arp2/3 complex via WASP (Srinivasan et al., 2003). Additionally Cdc42 orients the microtubule-organizing center and the Golgi apparatus in front of the nucleus, facing the leading edge. In this way Cdc42 promotes the delivery of Golgi vesicles through microtubules, providing membrane and proteins necessary for protrusion (Etienne-Manneville and Hall, 2002). Several evidences show that Cdc42 triggers filopodia formation, structures with parallel actin bundle organization that are believed to serve as sensors to explore the external environment. Recently it has been demonstrated that

Cdc42-induced filopodia are precursor-like structures for Rac-mediated lamellipodia (Guillou et al., 2008). Interestingly integrin occupancy plays a crucial role in this process.

RhoA regulates FAs assembly and cell contractility responsible for rear cell retraction during cell migration. RhoA regulates cell contractility by promoting the interaction between actin filaments and myosin. A crucial intermediate in this process is the Ser/Thr kinase p160ROCK. Indeed ROCK and RhoA are involved in single-cell migration process for their function in regulating cell rear detachment (Nobes and Hall, 1999). ROCK plays a role in cofilin-mediated stabilization of actin filaments (Maekawa et al., 1999). Additionally ROCK phosphorylates and inactivates myosin light chain (MLC) phosphatase. Phosphorylated myosin can cross-link actin filaments, leading to stress fiber formation (Mitchison and Cramer, 1996). β_3 integrin regulates Rho-mediated cell contractility through calpain cleavage. Indeed intact β_3 integrin interacts directly with Src and suppresses RhoA activity whereas proteolytic cleavage of its C-terminus abolishes Src interaction and promotes cell contractility (Flevaris et al., 2007). However β_1 integrin does not interact directly with Src (Arias-Salgado et al., 2005) and regulates RhoA in a different manner. Interestingly laminin binding to $\alpha_3\beta_1$ integrin suppresses RhoA activity and up-regulates cell migration while the $\alpha_2\beta_1$ integrin interaction with collagen stimulates RhoA activity and has an negative effect on cell motility (Zhou and Kramer, 2005). These evidences could indicate an additional regulatory role for the α subunits. Moreover, the expression level of different integrin types regulates the intensity of RhoA activation (White et al., 2007).

During cell migration and spreading the activity of Rho GTPases is tightly regulated. During initial cell adhesion and spreading RhoA activity is suppressed in favor of higher level of Rac1 and Cdc42 activation. This will result in low level of acto-myosin contractility and enhanced lamellipodia protrusion. After the first phase of cell spreading, Cdc42 and Rac1 activation decrease and concomitantly RhoA activity increases driving the

formation of actin stress fibers and the maturation of FAs. Interestingly RhoA and Rac1 suppress each other activity. This process is also crucial for the definition of the leading edge and the tail of the cell during cell migration. RhoA mediates the activation of Rac specific GAPs that favor the RAC-GDP bound (inactive) state (Ohta et al., 2006). Conversely Rac1 activation produces reactive oxygen species that activates p190RhoGAP, a Rho GAP that inhibits Rho activity (Nimnual et al., 2003).

Integrin-mediated regulation of signal transduction

The canonical way in which integrins signal is by engaging their specific ligands in the ECM. Integrin signaling can be divided in two types: transient and sustained. In transient signaling the signal decays after an initial peak in response to cell-matrix adhesion. Indeed cells seeded on ECM components display an increased SRC phosphorylation together with high Rac1 and Cdc42 activity that will decline to the baseline in few hours. Rho activity is initially strongly decreased. This initial phase is followed by a peak of activity and a subsequent decrease to the basal level. Sustained signaling is triggered by cell-matrix adhesion but, after the initial peak, remains elevated in time. A typical example is FAK auto-phosphorylation that is induced and maintained during cell-adhesion.

Several studies on the mechanism of outside-in signaling highlight the importance of the Src/FAK axis as major source of signal transduction pathways arising from integrins.

Src is a non-receptor protein kinase that together with fyn, yes, lyn (and other members expressed in specific cell types) compose the Src family kinase (SFK). All the SFKs members share a C-terminal tyrosine (Tyr529) that upon phosphorylation binds the

Src-homology 2 (SH2) domain, locking the molecule in an inactive conformation and inhibiting its kinase activity. Src activation is one of the earliest events following cell-adhesion and it is characterized by the dephosphorylation of the inhibitory Tyr529 in favor of the autophosphorylation of Tyr418. Src activation can be triggered by removal/inactivation of the inhibitory kinase Csk or by dephosphorylation of the inhibitory Tyr529 by integrin-associated phosphatases. Alternatively the activation of Src bound to integrin can occur through transactivation by itself or by competition of FAK for the SH2 domain (Mitra et al., 2005) (Arias-Salgado et al., 2005).

FAK is an ubiquitously expressed protein tyrosine kinase composed by a N-terminal FERM domain, a central kinase domain, proline rich regions and a C-terminal focal adhesion targeting (FAT) domain. The proline rich regions (PPR) are important in the interaction with Src homology 3 (SH3) containing proteins like p130Cas. Integrin ligation induces FAK autophosphorylation in Tyr397, creating an interaction site for the SH2 domain of Src. In this way, FAK competes for the binding to SH2 and stabilizes Src active conformation. Src in turn phosphorylates additional Tyr residues in FAK creating new docking sites for other proteins (Tyr861 and Tyr925) and increasing its kinase activity (Tyr576 and tyr577) (Mitra et al., 2005).

The active FAK-Src complex can activate Rac and trigger cell protrusion. A crucial adaptor protein in this process is p130Cas. FAK-Src complex activation results in p130Cas phosphorylation and the subsequent formation of a complex with v-Crk sarcoma virus CT10 oncogene homolog (CRK). CRK-p130cas complex in turn recruits Dock180 and engulfment and cell motility 1 (ELMO) (Chodniewicz and Klemke, 2004) that act as an unconventional GEF for Rac, promoting Rac-GTP active state (Brugnera et al., 2002). Additionally FAK-Src complex phosphorylates paxillin with consequent recruitment of ArfGAP paxillin-kinase linker (PKL) and Pak-interacting exchange factor beta (β-PIX a GEF for Cdc42) (ten Klooster et al., 2006). Alternatively FAK-SRC mediated paxillin

phosphorylation enables paxillin-Crk interaction (Turner, 2000). A schematic representation of FAK-Src role in signaling to Rho GTPases is given in Fig.10.

FAK-Src complex regulates also the transient repression of RhoA activity during the initial phase of cell spreading (Ren et al., 2000). Indeed $\alpha_5\beta_1$ integrin engagement triggers Src-mediated phosphorylation of p190RhoGAP with a consequent repression of RhoA contractile activity (Arthur et al., 2000).

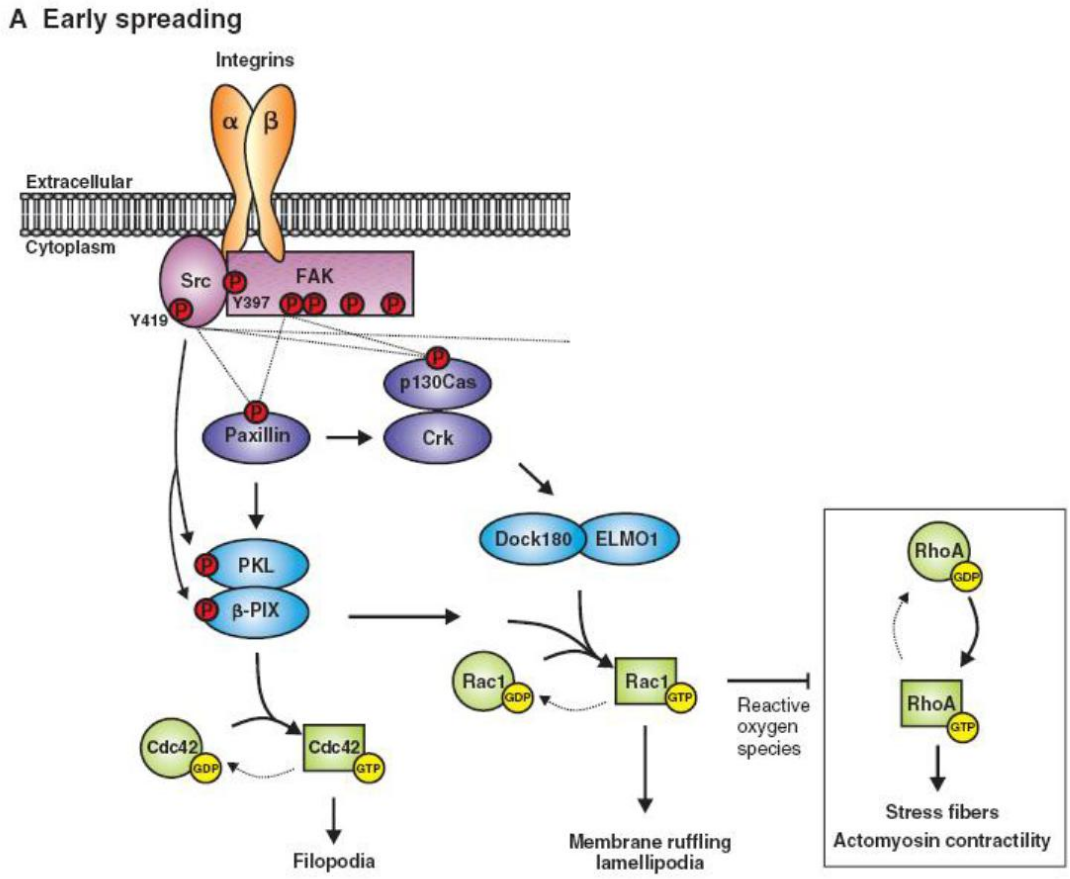


Figure 10 Early integrin signaling during cell spreading. Activated and clustered integrins recruits Src/FAK complex that upon activation phosphorylates paxillin and p130Cas. This lead to activation of Rac1 and Cdc42 and inhibition of RhoA (adapted from (Huveneers and Danen, 2009)).

After a first inactive phase, RhoA activation increases inducing actin stress fibers and FAs maturation. Rho GEF like p190RhoGEF, LARG and Gef1 are involved in this process (Dubash et al., 2007). Possibly mechanical signals could act locally and stimulate Src-dependent activation of these GEF. Several evidences indicate that $\alpha_5\beta_1$ integrin

binding to fibronectin is particularly efficient in promoting RhoA activation in the late phase of cell spreading (Danen et al., 2002). Conversely, the depletion of another fibronectin binding integrin, $\alpha_v\beta_3$, does not impair RhoA activation, although its overexpression can stimulate RhoA activity (Miao et al., 2002). Interestingly, $\alpha_5\beta_1$ integrin depletion results in low levels of RhoA activity and it is not rescued by expression of other FN binding integrins (Huveneers et al., 2008). RhoA activation could be induced by the mechanical perturbation of cyto-architecture in response to initial cell spreading. Consequently, mechanosensitive FAs protein can alter their conformation in response to mechanical stimulation. This event could expose cryptic binding sites for signaling molecules, resulting in differential signaling to Rho GTPases (Fig. 11)

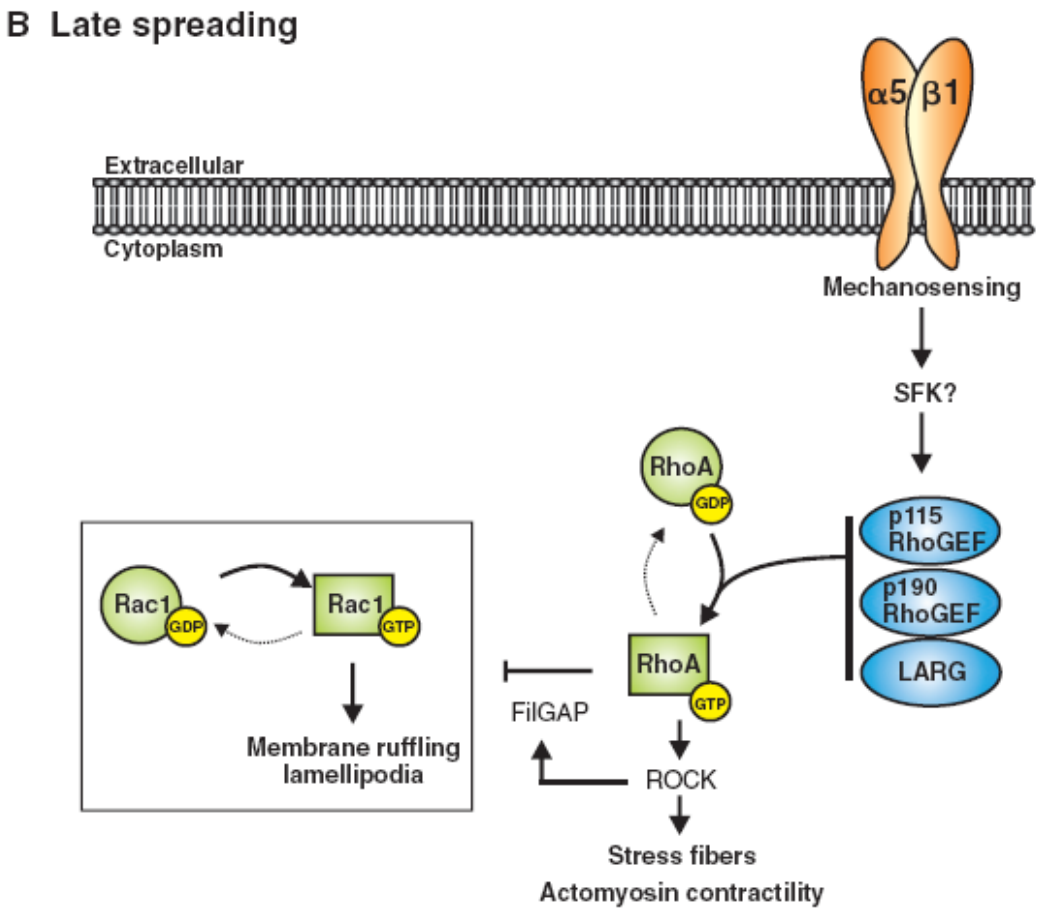


Figure 11 Late integrin signaling in cell spreading. Integrins activate SFKs, stimulating the activity of RhoA GEFs and enhancing RhoA activity.(adapted from (Huveneers and Danen, 2009))

These evidences indicate that Src-FAK complex can modulate and localize the activity of Rho GTPases, orchestrating cell protrusion dynamics in the different phases of cell adhesion, spreading and migration. Moreover Src directly interacts, through its SH3 domain, with the C-terminal part of β_3 integrin tail and this interaction has been shown to regulate cell spreading (Arias-Salgado et al., 2005). Src-β_3 integrin complex can phosphorylate Rac GEF, like Vav1, Vav2 or Tiam1, leading to Rac-dependent cell protrusions and spreading (Hamelers et al., 2005) (Marignani and Carpenter, 2001). Recently, the guanine nucleotide-binding protein (G protein) $G_{\alpha 13}$ has been shown to directly interact with β_3 integrin tail. This interaction is promoted by integrin ligation and by guanosine triphosphate (GTP) loading. $G_{\alpha 13}$-integrin interaction is crucial for Src activation and consequent RhoA inhibition in the early phase of cell spreading (Gong et al., 2010).

Beside the control of Rho GTPases activity, Integrins activate other signaling pathways. Mitogen activated protein kinases (MAPK) signaling pathway is activated in response to integrin ligation and modulates focal adhesion dynamic, proliferation, cell cycle progression and survival. Active Src phosphorylates FAK in Tyr925, creating a binding site for GRB2 adaptor protein. GRB2 recruits SOS, an exchange factor for Ras, leading to the activation of Ras. Active Ras can trigger the sequential activation of Raf and MEK that will result in ERK1/2 phosphorylation (Schlaepfer et al., 2004). Another way in which FAK can control MAPK activation is through the activation of PAK1, with consequent phosphorylation of MEK1 (Slack-Davis et al., 2003). Although MAPK activation can be induced by growth factors, integrin-mediated cell adhesion is required in order to get the complete activation of this signaling pathway. Finally, phosphorylated ERK1/2 activates myosin light chain kinase, modulating focal adhesion dynamics during cell motility (Ridley et al., 2003).

Integrin-mediated FAK activation allows also FAK interaction with PI3kinase. PI3kinase activation modulates cell survival through the AKT pathway and induces accumulation on PIP3 lipid messenger in the leading edge of the cells (Sonoda et al., 1999).

Integrin can activate also the PKC Ser/Thr kinases signaling pathway. Indeed PKC members are activated as long as the cells are adhering through integrins (Ivaska et al., 2003). PKCα has been reported to regulate integrin trafficking by directly interacting with β_1 integrin in active state (Ng et al., 1999).

An alternative way in which integrins influence intracellular signaling is by modulating the recycling of lipid raft, regulating in this way the membrane order. Indeed cell detachment from the ECM triggers lipid rafts clearance in a process that requires caveolin 1 phosphorylation in tyrosine 14 and dynamin-2. This process regulates the dependency on cell adhesion of most of the signaling pathways. Consistently caveolin -/- MEFs loose the dependency on cell adhesion in the activation of AKT, ERK1/2 and PAK signaling pathways (del Pozo et al., 2005). Moreover lipid rafts internalization that follows cell detachment inhibits Rac1 membrane targeting and, in this way, its activation (del Pozo et al., 2004). When integrin-ECM contacts are reconstituted by replating the cells on fibronectin, lipid rafts exit from recycling endosomes in an Arf6 dependent manner and return to the plasma membrane along microtubules (Balasubramanian et al., 2007). Through this mechanism integrin can control signal transduction by regulating membrane composition. In this way the function of proteins that need, beside an active state, membrane targeting (like Ras, Rac and Src) will be dependent on integrin-mediated cell adhesion.

Finally, integrins are involved in many mechanotransduction processes in a direct or indirect way. Cells can sense through integrins changes and deformations of the extracellular matrix, modification of hydrostatic pressure, fluid shear stress and osmotic

forces. Mechanical stimuli, as the stretch of the ECM, can change the geometry and composition of focal adhesions by exploiting the straight connection among ECM, integrin and the cytoskeleton. In the case of osmotic or hydrostatic pressure, the way in which integrins are involved is not clear. Several evidences indicate that applied forces or increased cellular contractility result in enlargement of FAs. In this way forces acting directly on adhesion sites can promote focal complexes to focal adhesion transition and modulate FAs dynamics. Increased tension on integrin leads to the recruitment of vinculin and other focal adhesion components. Indeed by stretching the talin rod, a cryptic vinculin binding site is unmasked (del Rio et al., 2009). Additionally tension triggers the activation of unoccupied/inactive integrins, with consequent ECM ligation (Katsumi et al., 2005). In this way, cells can adjust their adhesive force in response to variation in their extracellular context. Moreover several evidences show that mechanical strain on adherent cells activates FAK and Src (Plotkin et al., 2005). Thus, ECM strain induces integrin activation and focal adhesion strengthening leading to signal transduction. Integrin-mediated mechanotransduction is thought to involve proteins that undergo conformational changes under force. A typical example is p130Cas that, in response to force, assumes an open conformation allowing substrate domain phosphorylation (Sawada et al., 2006). Interestingly integrins are implicated in the mechanotransduction process also in system where forces are not directly acting on them. Osmotic stress responses allow cell to adjust to changes in ion transport or in extracellular osmolarity. Osmotic stress induces Src and FAK phosphorylation in a integrin dependent manner (Browe and Baumgarten, 2003). Moreover cell stretching induces the opening of stretch-activated ion channels in an integrin dependent fashion (Miyauchi et al., 2006). Cells can also respond to changes in hydrostatic pressure. A small elevation of pressure activates integrins and cause the phosphorylation of Src, FAK and α-actinin. Interestingly pressure-mediated effect occurs also in suspended cells, where integrins do not engage their ligands (Craig et al., 2007).

Integrins and cell migration:

Integrins are the major family of migration-promoting receptors. First integrins provide stable adhesion to the ECM, acting as the feet of migrating cells. Second integrins form an uninterrupted connection between ECM and cytoskeleton. Third integrin transmit a wide array of signals implied in cell polarity, cell contraction and cell protrusion.

Integrins form adhesion sites at the leading edge on the cells. The multivalent properties of ECM components as well as Cdc42 and Rac activity are involved in this process (see below). Adhesion formation mediates attachment to the ECM and stabilizes lamellipodia protrusion with the onset of positive feed-back loops. During cell migration focal complexes maturation and assembly are strictly regulated, as cells with large focal adhesion are generally immobile.

During migration, cells have to detach their rear to allow an efficient translocation of the cell body but at the same time they have to exert traction of the ECM. Thus, the strength of cell adhesion modulates cell motility and it is influenced by the density and the topology of ECM ligands, the amount and the type of integrin receptors on the cell surface and the activation state of adhesion molecules. Myosin II is implicated in cell contractility and it has a primary role in the transmission of force to the adhesion sites (Mitchison and Cramer, 1996).

In migrating cells, the major forces are transmitted to the focal complexes in the leading edge and to the retracting cell rear (Beningo et al., 2001). In the leading edge the adhesion sites under cell protrusion disassemble in favor of newly formed ones (Webb et al., 2002). However, some adhesions maturate into more stable FAs. One possible regulatory mechanism is microtubule targeting with consequent FAs disassembly (Small and Kaverina, 2003).

Many proteins with different functions regulate the adhesion turnover in the leading edge. FAK and Src depleted cells are characterized by slow migration speed and large FAs (Webb et al., 2002). FAK/Src/p130Cas-mediated Rac activation is crucial for cell protrusion and focal adhesion turnover. Additionally Rac-mediated Rho suppression and MAPKs activation has been reported to play a crucial role in this process. Finally proteolytic cleavage of integrins and FAs components by calpain is yet another way in which focal adhesion disassembly is regulated in the leading edge (Franco et al., 2004).

The rear of the cell is characterized by a dynamic focal adhesions disassembly. In many cells types, cell adhesion is strong at the rear resulting in an elongated morphology characterized by a long migration tail. The tension exerted on the adhesion sites could be sufficient to break integrin-cytoskeleton linkage with a consequent translocation of the cell body. Indeed the tension in the rear of the cells contributes to its detachment (Lauffenburger and Horwitz, 1996). Myosin II contractile function is a crucial element in this process. Moreover, the FAK/Src axis is implicated also in focal adhesion disassembly in the rear of the cells. Finally, FAs disassembly is stimulated by calcium influx. Indeed the elevated membrane tension during cell migration could trigger the opening of stretch-activated ion channel with consequent increase of the intracellular calcium concentration (Lee et al., 1999).

NON-INTEGRIN CELL ADHESION

Integrins are the main transmembrane receptors that mediate cell adhesion to the ECM and subsequent signaling. Besides integrins, other adhesion molecules mediate cell adhesion and signaling directly or in co-operation with integrins. Among them CD44 provides high affinity adhesion to ECM ligands by binding glycoproteins, glycosaminoglycans and hyaluronic acid (HA) (Ponta et al., 2003). CD44 binds its main ligand, HA, through hyaluronan-binding domain (Banerji et al., 2007). Additionally CD44 interacts also with non-protein ECM ligands like heparan sulphate, chondroitin sulphate and with canonical ECM proteins like collagen type 1 and VI, fibronectin and laminin (Ponta et al., 2003). CD44 possesses a cytoplasmic domain that interacts with actin binding proteins like ezrin, radixin, moesin , mediating the connection with the actin cytoskeleton (Bourguignon, 2008). Additionally CD44-mediated HA adhesion transduces different signaling pathways that involve Src (Ouhtit et al., 2007), Rac1 and RhoA (Bourguignon et al., 2000). RhoA activation recruits, through its effector ROCK, ankirin to the CD44 cytoplasmic tail, providing a second indirect link with the actin cytoskeleton (Bourguignon et al., 2004).

HA can be associated to tissues but can be also immobilized at the cell surface by CD44 interaction (Rilla et al., 2008). Several evidences indicate that membrane-associated HA engages other ECM components and it is localized at the tips of cell protrusion. Surprisingly HA-ECM interaction precedes the formation of integrin-dependent focal

contacts. Thus CD44-mediated HA membrane localization and the following HA-ECM engagement could represent a very early event in ECM-cell interaction (Zimmerman et al., 2002). CD44 enhances cell migration by increasing cell-matrix interaction or by enhancing pro-migratory signals (Zhu et al., 2006). Interestingly during cell migration CD44 does not localizes in the leading edge but rather uniformly interacts with the ECM with an enrichment in the cell rear (Jacobson et al., 1984). CD44-HA interaction can activate extracellular proteolysis which result is cleavage of its ectodomain with consequent release of its adhesive bonds (Nagano et al., 2004). However the *in vivo* relevance of CD44-mediated adhesion remains unclear as CD44 deficiency does not result in any major phenotype in mice (Protin et al., 1999).

Besides CD44, the syndecans family of cell surface heparan sulphate proteoglycans mediate non-integrin cell adhesion. Their heparin-binding ectodomain allows syndecans interaction with extracellular glycosaminoglycans and ECM proteins such as collagens, FN and VN (Beauvais and Rapraeger, 2004). Syndecans overexpression usually increases cell adhesion and migration in normal and cancer cells whereas their down-regulation has an inhibitory effect (Beauvais and Rapraeger, 2004). However, the strong synergy with integrins frequently masks the direct contribution of syndecans to cell adhesion and migration.

A third family of non-integrin adhesion receptors is composed by discoidin domain receptors (DDR) that can mediate adhesion to fibrillar collagens. DDR-mediated cell adhesion induces intracellular signaling pathways that promote actin dynamics (Vogel et al., 2006). Interestingly, DDR signaling occurs after few minutes but displays a peak after several hours. This evidence could indicate that DDR are not involved in early and acute responses to the ECM but they play a function in sustained and slow responses (Vogel et al., 2006). In overexpression models, DDRs induce collagen adhesion directly and independently of integrins (Kamohara et al., 2001). However, DDR signaling occurs in

close co-operation with integrins. Indeed DDRs enhance integrin-mediated adhesion and, in this way, reinforce integrin-dependent signal transduction (Shintani et al., 2008).

Finally, urokinase plasminogen activator receptor (uPAR) interacts with the N-terminal portion of VN, inducing integrin-independent adhesion to this ECM component. The mechanism of uPAR-mediated cell adhesion and signaling will be extensively analyzed in the following chapter.

uPAR AND THE PLASMINOGEN ACTIVATION SYSTEM

Urokinase plasminogen activator (uPA) receptor (uPAR) is an extensively N-glycosylated membrane receptor tethered to the plasma membrane by a glycosyl-phosphatidylinositol (GPI) anchor. uPAR modulates pericellular proteolysis by regulating the activity of the plasminogen activation (PA) system. The binding of its natural ligand, the serine protease uPA both in its zymogen or activated form, localizes and concentrates this protease activity on the cell surface. Active uPA in turn proteolytically activates the zymogen plasminogen, generating the active protease plasmin. The uPA activation process is proteolytically regulated as well and plasmin establishes a positive feedback loop that increase uPA activity (Ellis et al., 1991). The plasminogen activation system is negatively regulated by plasminogen activation inhibitors 1 and 2 (PAI1 and PAI2) which covalently bind to their target and induce the internalization of the ternary complex (uPAR:uPA:PAI1) through low-density lipoprotein receptor related complex (LRP) (Cubellis et al., 1990). Active plasmin can directly cleave a wide range of ECM components, playing a crucial role in fibrin clots clearance (fibrinolysis). Alternatively plasmin activates other protease activities

like metallo-proteases (MMPs) amplifying the proteolytic cascade (Carmeliet et al., 1997). In this way uPAR localizes PA in the leading edge of the cells facilitating migration through three-dimensional ECM (Estreicher et al., 1990). (Fig. 12)

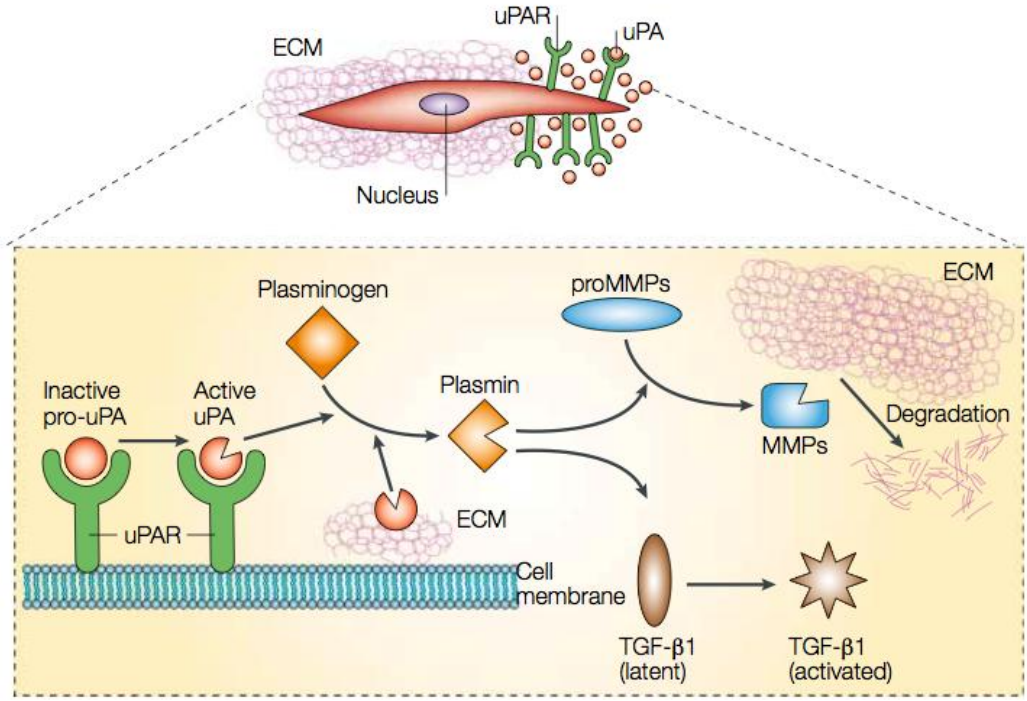


Figure 12: The plasminogen activation system. uPA-uPAR complex catalyzes the conversion of plasminogen in to plasmin. Active plasmin can activate MMPs, directly degrade ECM or activate latent growth factors (adapted from (Blasi and Carmeliet, 2002)).

Interestingly under physiological conditions, uPAR expression is rather low whereas it is increased with the onset of pathological conditions like cancer. Indeed increased levels of uPAR expression correlates with poor prognosis, cancer progression and metastatic dissemination (Sidenius and Blasi, 2003).

Besides its role in pericellular proteolysis, uPAR regulates cell adhesion and signal transduction pathways modulating cell migration and proliferation. Adhesive, proteolytic and signaling functions are often linked together. By degrading the ECM, uPAR release

and activate growth factors (Houck et al., 1992). Moreover uPAR mediates directly cell adhesion to ECM protein vitronectin (VN) (Wei et al., 1994) and several evidences indicate uPAR-VN interaction to have a crucial role in signal transduction (Madsen et al., 2007) (Kjoller and Hall, 2001) (Smith et al., 2008). Interestingly uPAR does not possess a cytoplasmic domain, being a GPI molecule. The mechanisms of uPAR-mediated signal transduction are controversial and seem to involve functional interactions with other transmembrane receptors.

uPAR structure:

uPAR is a 283 aa GPI-anchored protein composed by three consecutive three-finger domains: DI (residues 1-80), DII (residues 93-191) and DIII(residues 192-283). DI and DII contain six β-strands while DIII only five. Moreover, uPAR aminoacidic sequence contains 28 cysteines that create 4-5 inter-chain disulphide bonds in each domain (Huai et al., 2006). In the mature receptor, the three domains dispose themselves in a circular fashion generating central cavity in which uPA accommodates. uPA interacts with uPAR through its aminoterminal growth factor domain (GFD) and also the aminoterminal fragment (ATF that contains only growth factor and kringle domain but not the protease domain) of uPA has been shown to be sufficient for the interaction with uPAR (Appella et al., 1987).The inter-domain interfaces are essential regions that support the globular structure of the receptor. The non-covalent interaction among inter-domain interfaces provides flexibility and allows uPA to trigger modification in the receptor structure (Huai et al., 2006). In particular, the structure of the individual domains is unchanged whereas the orientation of the three domains is significantly altered (Huai et al., 2006). In addition,

the linker region connecting DI to DII is modified upon uPA interaction and it is immobilized in its open conformation (Barinka et al., 2006).

uPAR is heavily glycosylated, containing 5 potential N-glycosylation sites (Asn52, Asn162, Asn172, Asn200 and Asn 233) although studies on CHO cells demonstrated that only the first four are used (Ploug et al., 1998). uPAR glycosylation profile is rather heterogeneous and depends on the cell type and differentiation state (Lund et al., 1995).

The uPAR GPI-anchor is attached to Gly283 and plays an important role in membrane localization. Phospholipases can cause the release of the receptor from the plasma membrane, generating in this way a soluble uPAR (suPAR) variant that accumulates in blood and urine (Wilhelm et al., 1999). GPI-anchor enables uPAR association with membrane microdomains enriched in cholesterol and sphingolipids termed lipid rafts. Lipid rafts partition is strictly linked to uPAR dimerization in a process stimulated by uPA interaction. In particular uPA induces uPAR dimerization and dimeric uPAR preferentially associates with lipid rafts (Cunningham et al., 2003). The fact that dimeric uPAR has increased VN affinity indicates a putative mechanism through which uPA increases uPAR-mediated VN adhesion (Sidenius et al., 2002) (see below). Moreover, lipid rafts are hot spots in the plasma membrane where multiple protein-protein interactions between transmembrane receptors take place. Indeed the signaling from uPAR requires lipid raft partition, although their detergent-resistant nature and the elevated molecular crowding that characterizes them make immune-precipitation and co-localization assays unreliable.

The linker region connecting DI and DII is susceptible to cleavage by proteases like uPA itself (Hoyer-Hansen et al., 1992), plasmin, and MMPs (Andolfo et al., 2002). uPAR cleavage release DI from DII-DIII, that remains anchored to the plasma membrane. Cleaved uPAR loses the capacity to interact with uPA and thereby it cannot support plasminogen activation (Hoyer-Hansen et al., 1992). Additionally DI is crucially involved

in uPAR-mediated VN adhesion (Hoyer-Hansen et al., 1997) and the intact uPAR molecule is required for the interaction with other membrane receptors (Montuori et al., 2002). These evidences suggest that uPAR cleavage regulates its signaling capacity in a negative way. Moreover uPA bound to uPAR can cleave other neighbor uPAR molecules with consequent increase of uPAR cleavage occurring in lipid rafts (Cunningham et al., 2003). However, uPAR cleavage unmask a chemotattic epitope located in the linker region between DI and DII. When shed from the cell surface DIIDIII interacts with formyl peptide receptor-like 1 (FPRL1), a G-protein coupled receptor (GPCR), and induces chemotaxis of monocytes (Resnati et al., 2002).

uPAR-vitronectin interaction

Vitronectin is an extensively glycosylated multifunctional ECM components initially termed as "serum spreading factor". It reaches high concentration as monomer in the blood stream (up to 200-500 μg/ml) and it is converted to a multimeric form by incorporation into the ECM (Hayman et al., 1983). Multimeric VN exposes binding sites specific for integrins ($\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_v\beta_1$ and $\alpha_{IIb}\beta_3$) , uPAR and PAI-1 (Preissner and Seiffert, 1998).VN is found in many organs, blood vessels and lymph nodes and increased VN deposition has been observed in many tumors (Loridon-Rosa et al., 1988).

VN is composed by a N-terminal somatomedin-B domain (aa 1-44) followed by a specific integrin binding site (RGD motif aa 45-47), an highly acidic region and a collagen binding domain. The remaining part of the molecule is composed by hemopexin-type domains involved in the oligomerization of the protein (Chillakuri et al., 2010) and in the

binding of heparin, glycosaminoglycans, collagen and plasminogen (Schvartz et al., 1999).

Interestingly uPAR directly interacts with VN (Wei et al., 1994). In this way, uPAR can modulate cell adhesion independently of integrins. Indeed uPAR can support VN adhesion even on a vitronectin variant with disrupted RGD motif (Madsen et al., 2007). Consistently EDTA, RGD-peptides or integrin blocking antibodies do not impair uPAR-induced VN adhesion (Wei et al., 1994).

uPA plays a role in this process presumably for its effect on uPAR structure, dimerization and lipid raft partition. Indeed uPAR bound to uPA, or the catalytically inactive ATF, has increased affinity for VN (Sidenius et al., 2002). In cells expressing low level of receptor VN adhesion requires uPA binding while high levels of expression induce cell adhesion independently of receptor occupancy. The epitope in uPAR responsible for VN binding has been mapped and it is composed by three aminoacids from DI (Trp32, Arg58, Ile 63) and two aminoacids from DII (Arg91 and Tyr92) (Gardsvoll and Ploug, 2007; Madsen et al., 2007). The crucial contribution of DI in forming the VN-binding epitope explains why uPAR cleavage abolishes uPAR-VN interaction (Sidenius and Blasi, 2000). Interestingly the uPA and the VN binding site do not overlap, being the first one located in the top/back part of the molecule whereas the second one lies in the central cavity (Fig.13). This evidence explains how uPAR engages at the same time both uPA and VN (Huai et al., 2006).

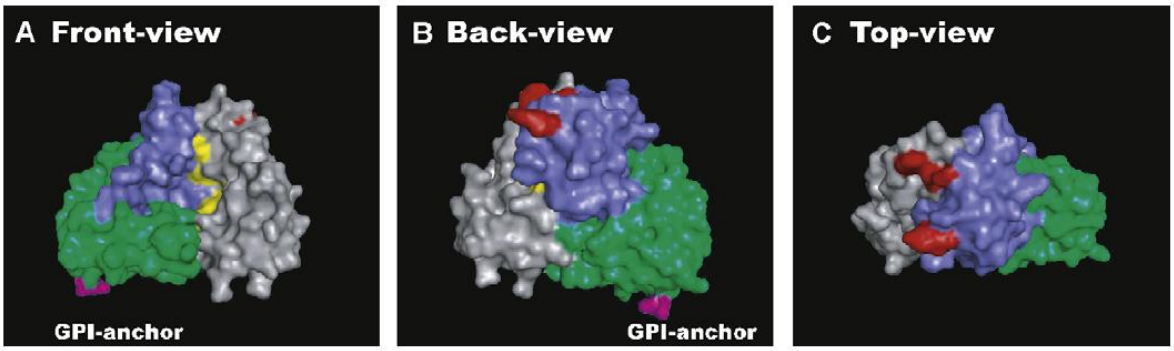


Figure 13: Crystal structure of uPAR. The three domain are indicated in gray (DI), blue (DII) and green (DIII). The central cavity that constitutes uPA-binding site is visible in the front view. The residues involved in uPA interaction are colored in yellow. Back and top view show the VN-binding epitope, whose residues are indicated in red. GPI anchor location is indicated in magenta (adapted from (Madsen and Sidenius, 2008)).

The uPAR-binding site on VN is located in the SMB domain. Indeed the deletion of SMB abolishes uPAR-VN interaction (Madsen et al., 2007). Interestingly the same epitope used by uPAR is also the binding site for PAI-1 and comprises residues Asp22, Leu24, Tyr27 and Tyr28 (Zhou et al., 2003). Several structural and functional evidences suggest that PAI-1-VN interaction may sterically interfere with integrin binding (Zhou et al., 2003).

Mechanism(s) of uPAR-mediated signaling

uPAR lacks a cytoplasmic domain and therefore it is a signaling incompetent molecule *per se*. However, uPAR expression is reported to dramatically alter the intracellular signaling by functionally or directly coupling to other transmembrane receptors. uPAR can transduce a signal through GPCRs and RTKs like platelet derived growth factor receptor-β (PDGFRB) and epidermal growth factor receptor (EGFR). However, the major transmembrane receptors through which uPAR transmit a signaling are integrins. The inner

nature of uPAR-integrins cross-talk remains controversial however a growing number of evidences show that uPAR requires integrins in order to signal. In many studies uPAR has been found to co-localize or co-immunoprecipitate with integrins (see below), although direct protein-protein interaction assays failed in detecting a direct interaction between uPAR and integrins (Bass and Ellis, 2009).

The first integrin found to interact with uPAR is MAC1 ($\alpha_M\beta_2$) (Xue et al., 1994), an integrin expressed predominantly in leukocytes. uPAR co-localizes and co-immunoprecipitates with MAC1 (Bohuslav et al., 1995), regulating fibrinogen binding, promoting VN adhesion (Simon et al., 1996) and inducing chemotaxis (Gyetko et al., 1994) in a Src dependent manner (Bohuslav et al., 1995).

A functional cross-talk was also found between uPAR and $\alpha_5\beta_1$ integrin with a consequent modulation of integrin adhesive capacity. In particular uPAR-integrin interaction converts the canonical RGD-dependent FN adhesion into a RGD-independent binding. Mutations on β_1 integrin (Ser227Ala) or in uPAR (His249Ala) abolish this interaction and uPAR-mediated effects (Wei et al., 2005) (Wei et al., 2007). Additionally, a peptide derived from β_1 integrin interaction site blocks uPAR-integrin interaction (Wei et al., 2005). RGD-independent FN adhesion is required for FAK/Src-dependent Rac1 activation (Wei et al., 2007). These evidences are in contradiction with other studies where uPAR association with $\alpha_5\beta_1$ integrin induces RGD-dependent FN adhesion and generate a constitutively mitogenic signal that suppresses tumor dormancy, stimulating tumor growth. Moreover the residue in uPAR involved in the direct interaction with β_1 integrin differs from the one described above, being Ser245 (Chaurasia et al., 2006).

uPAR-β_1 integrin complex can also signal through EGFR. Indeed uPAR-integrin interaction has been shown to induce EGFR activation through FAK (Liu et al., 2002). Moreover uPAR expression results in EGFR phosphorylation in Tyr845, leading to increased proliferation upon EGF stimulation (Jo et al., 2007). Interestingly EGFR

inhibitors did not impair uPAR-induced Rac activation indicating that different pathways can be transduced by uPAR at the same time (Jo et al., 2003).

An additional interaction was found between uPAR and $\alpha_3\beta_1$ integrin. This interaction activates $\alpha_3\beta_1$ integrin allowing VN adhesion (Wei et al., 2001). Additionally uPAR-$\alpha_3\beta_1$ integrin interaction was found to trigger epithelial-to-mesenchymal transition in epithelial cells (Zhang et al., 2003).

Several synthetic peptides have been used to disrupt uPAR-integrin interaction. A peptide (p25) selected from a phage display was found to inhibit uPAR/β_1 integrin interaction, blocking both uPAR-$\alpha_5\beta_1$ integrin co-immunoprecipitation (Wei et al., 1996) and $\alpha_3\beta_1$ integrin-mediated VN adhesion (Wei et al., 2001). Subsequently, a linear sequence within MAC1 was identified as a crucial uPAR interaction sites. A synthetic peptide derived from this sequence (M25) was shown to inhibit uPAR interaction with a subset of β_1 and β_2 containing integrins (Simon et al., 2000).

Finally uPAR interacts with β_3 integrin, inducing Rac1 activation and lamellipodia protrusion. Lipopolysaccharide treatment induces uPAR expression in kidney podocytes, leading to $\alpha_v\beta_3$ integrin activation and Rac-dependent cell motility (Wei et al., 2008). Moreover uPAR-β_3 integrin interaction in tumor cells triggers the activation of the Src/p130Cas/Rac signaling pathway that regulates cancer cell invasion (Smith et al., 2008).

However, the existence of a direct-lateral interaction between uPAR and integrins is not directly proved by co-immunoprecipitation, co-localization experiments or by the inhibitory effect exerted by peptides. Indeed the relevance of direct uPAR interaction(s) with transmembrane receptors has been questioned by a complete alanine scanning of the entire receptor. In this study, mutations in residues involved in uPAR-mediated VN adhesion impaired lamellipodia protrusion and morphology changes in two different cell systems. Mutations in the remaining residues, and especially in the previously published

integrin binding sites (even combined together), produced receptors whose function was indistinguishable from the WT one. Finally, the expression of an artificial VN receptor, consisting in PAI-1 tethered to the plasma membrane by a GPI-anchor, recapitulated uPAR-VN signaling and morphological changes. As PAI-1 and uPAR shares no sequence homology, this indicates that uPAR-mediated VN adhesion is required and sufficient to induce changes in cell morphology and cell migration. Even if uPAR physically associates with integrins or other transmembrane receptors, these direct interactions are dispensable for uPAR-mediated effects (Madsen et al., 2007). Moreover, uPAR-VN interaction is required for rearrangement of FA components and actin cytoskeleton in fibroblasts (Kjoller and Hall, 2001). Other studies indicate that uPAR-VN interaction is required for signaling and cancer cell migration even if it cannot be detected in standard adhesion assays (Smith et al., 2008). These evidences can lead to an alternative mechanism of signal transduction by uPAR. uPAR-VN interaction could bring integrins closer to the ECM, facilitating integrin engagement and thus modulating integrin signaling/activation by increasing the cell-matrix contact area. This model would explain how uPAR could functionally interact contemporaneously with so many different integrin types, attributing uPAR-specific effects to the integrin ligands present in the ECM and to the integrin expression level on the cell surface. The mechanism behind this process could be envisioned like a real co-operation between different adhesion receptors where uPAR amplifies integrin signaling by increasing cell adhesiveness. Additionally VN could be the bridge connecting uPAR and integrins, explaining uPAR-integrin co-immunoprecipitation (especially in experiments performed in low stringency conditions). Indeed, in many studies on uPAR-integrin interaction, the experiments are performed culturing the cells in serum condition, which contains high amount of VN. Finally many cell lines efficiently adhere to VN through integrins, masking an eventual contribution by uPAR while in cell lines adhering poorly to VN the uPAR-specific effect on cell adhesion could be better appreciated.

Interestingly FRET analysis on different ECM components shows that in cell seeded on VN, uPAR localizes in the basal membrane in a dimeric and low diffusing form. Consistently, uPAR differential localization is lost on FN (Caiolfa et al., 2007). This evidences could implicate that uPAR (presumably in a dimeric form) interacts with VN and generate "hot adhesive spots", characterized by a closer proximity to the ECM. Integrins close to/inside these adhesive spots will be forced to engage their ECM components, increasing their signaling activity. Moreover the mechanical interaction with the ECM could generate forces that could act on pre-engaged integrin, leading to mechanotransduction.

AIM OF THE WORK

The aim of this work is to elucidate the molecular mechanism of uPAR/VN interaction signaling through structure-functional analysis of uPAR, VN and the signaling receptor(s) involved in this process.

MATERIAL AND METHODS:

Cloning description

β_1 integrin oligos and cloning:

To generate Flp-In expression vectors for the B1-integrin (i.e.) a β1A cDNA (OpenBiosystems, Cat# MHS1010-58245) was amplified with oligos B1f/B1ageR (product digested KpnI/AgeI) and B1tmF/B1r (product digested AgeI/NotI) and assembled in KpnI/NotI digested pcDNA5/FRT/TO (Invitrogen corp.) generating pFRT/TO-$\beta1^{WT}$. This procedure introduces a unique AgeI restriction site by silent mutagenesis allowing for the easy swapping of the extracellular and membrane/intracellular coding regions between different constructs. The single substitution mutants $\beta1^{763A}$, $\beta1^{763F}$, $\beta1^{775A}$ and $\beta1^{775F}$ were generated by amplification of pFRT/TO- $\beta1^{WT}$ with oligos B1tmF and B1Y763Ar, B1Y763Fr, B1Y775Ar or B1Y775Fr followed by re-cloning of the products AgeI/NotI in the parental vector. The single amino acid substitution mutants $\beta1^{218A}$, $\beta1^{130D}$ and $\beta1^{227A}$ were generated by site-directed mutagenesis using oligos B1K218f/r, B1D130f/r and B1S227f/r, respectively. Constructs containing multiple substitutions were generated by multiple rounds of mutagenesis.

B1f 5’-cggggtacccgccgcgcggaaaagatgaatttacaaccaattttctgg-3’

B1ageR 5’-ggaccggtgggacactctggattctc-3’

B1tmF 5’-ccaccggtccagacatcattccaattgta-3’

B1r 5’-tgcgcggccgctcattttccctcatacttcggattgacca-3’

B1K218f 5’-aatgaacttgttggagcacagcgcatatctgga-3’

B1K218r 5’-tccagatatgcgctgtgctccaacaagttcatt-3’

B1D130f 5’-ctctactaccttatggccctgtcttactcaatg-3’

B1D130r 5’-cattgagtaagacagggccataaggtagtagag-3’

B1S227f 5’-tctggaaatttggatgctccagaaggtggtttc-3’

B1S227r 5’-gaaaccaccttctggagcatccaaatttccaga-3’

B1Y763Ar 5’-
tgcgcggccgctcattttccctcatacttcggattgaccacagttgttacggcactcttagcaataggattttcacc-3’

B1Y763Fr 5’-
tgcgcggccgctcattttccctcatacttcggattgaccacagttgttacggcactcttaaaaataggattttcacc-3’

B1Y775Ar 5’-tgcgcggccgctcattttccctcagccttcggattgacca-3’

B1Y775Fr 5’-tgcgcggccgctcattttccctcaaacttcggattgacca-3’

β_3 integrin oligos and cloning:

The expression vector for B3-integrin (pFRT/TO-B3) was generated by amplifying a human B3 cDNA (OpenBiosystems, Cat# MHS4426-99626129) with oligos B3fn4/B3tmcR (product digested HinDIII/AgeI) and B3tmcF/B3rxx (product digested AgeI/XhoI) and assembling the fragments in HinDIII/XhoI digested pcDNA5/FRT/TO. This procedure introduces a unique AgeI restriction site allowing for the easy swapping of the extracellular and membrane/intracellular coding regions between different constructs. The introduction of this restriction site causes a single amino acid substitution ($K^{689}T$) that apparently does not compromise receptor function. The $D^{119}Y$ mutation in pFRT/TO-$B3^{119Y}$ was generated by site-directed mutagenesis using oligos B3119Yf/B3119Yr. Mutations preventing the interaction with intracellular adaptor proteins ($Y^{747}A$, $S^{752}P$ and $Y^{759}A$) were introduced by amplification of pFRT/TO-B3 with oligos B3tmcF/B3YSYrx and re-cloning the product AgeI/XhoI in the parental vector.

B3fn4 5’-aaaaagcttccaccatgcgagcacggccgcggcccc-3’

B3tmcF 5’-cc<u>accggt</u>cctgacatcctggtggtcctgctc-3’

B3tmcR 5’-tgcgcggccgcttaagtgccccggtagctgat-3’

B3rxx 5’-tgcctcgagttaagtgccccggtagctgat-3’

B3119Yf 5’-atctactacttgatgtacctgtcttactccatg-3’

B3119Yr 5’-catggagtaagacaggtacatcaagtagtagat-3’

B3YSYrx 5’-

gcctcgagttaagtgccccgggccgtgatattggtgaaggtgggcgtggcctctttagccagtgggttgtt-3’

<u>VN cloning:</u>

A human VN cDNA (RZPD Clone ID: IRAUp969G1135D6)) was amplified with oligos hVNu(Bam)/hVNd(Xba) and the PCR product cloned BamHI/XbaI in pBluescript. A 6xHis tag was introduced at the C-terminal by digestion with XbaI/NotI and insertion of a linker made by annealing oligos XbNhisf and XbNhisR. The 6xHis tagged VN coding region was transferred BamHI/NotI to pcDNA5/FRT-TO generating the expression vector pFRT/TO-VN. The expression vector encoding VN lacking the SMB domain (pFRT/TO-VNΔSMB) was generated by amplification of pFRT/TO-VN with oligos SigUd40 and re-cloning the product BamHI/NotI. This strategy replaces amino acids 2-40 of VN with a single leucine residue.

HVnu 5’-gcggatccagccctgccatggcacccctgag-3’

XbNhisF 5’-ctagagggcatcatcaccatcaccattgagc-3’

XbNhisR 5’-ggccgctcaatggtgatggtgatgatgccct-3’

SigUd40 5’-cggggtaccatggcacccctgagacccccttctcatactg gccctgctggcatgggttgctctggctgacctcccccaagtgactcgcgggg-3’

RADf 5’-ccccaagtgactcgcgcggatgtgttcactatg-3’

RADr 5’-catagtgaacacatccgcgcgagtcacttgggg-3’

Materials

HEK293 Flp-In T-REx cells, expression vectors pcDNA5/FRT/TO and pOG44, zeocin, blasticidin S HCl and F-12 (Ham) medium were from Invitrogen. Dulbecco’s modified eagle medium (DMEM) is from Lonza. PBS, trypsin, glutamine, penicillin and streptomycin were obtained from EuroClone, while fetal bovine serum (FBS) was from HyClone. Non-tissue culture plates were from Falcon Becton Dickinson. Tetracycline, poly-L-lysine, laminin (from Engelbreth-Holm-Swarm murine sarcoma), anti-vinculin antibody (hVIN-1) and CHO protein-free culture medium were from Sigma. Fugene 6, fibronectin and Hygromycin B were from Roche. Pro-uPA was kindly provided by Dr. Jack Henkin (Abbott Laboratories). Antibodies against phosphorylated p130Cas, total and phosphorylated ERK1/2 were from Cell Signaling Technology. Blocking antibodies against $\alpha_v\beta_3$ (LM609) and $\alpha_v\beta_5$ (P1F6) integrins were from Immunological Sciences. Monoclonal antibody against β_1 integrin (mAb 13) was from BD Pharmingen. Monoclonal antibody against β_1 integrin (4B4) was from Beckam Coulter. Anti human uPAR R4 antibody was kindly provided by Dr. Gunilla Høyer-Hansen (Finsen Laboratory, Denmark). Glass bottom 12-well plates used for DIC and Timelapse microscopy are from

MatTek Corporation. Src inhibitors PP1, PP2 and PP3 are from Calbiochem. MEK inhibitor UO126 is from cell signaling. EGFR inhibitor AG1478 is from SIGMA. Dynabeads M-450 tosylactivated and the magnet used for the experiments were from Invitrogen.

Cell culture and transfection

293 Flp-In T-Rex cells were grown in DMEM supplemented with 10% FBS, penicillin 100 U/ml, streptomycin 100 U/ml, L-glutamine 5mM, 15 μg/ml blasticidin and 100 μg/ml zeocin at 37° in 5% CO_2. the Flp-In system generates pools of isogenic transfectants carrying a single copy of the expression cassette in exactlt the same chromosomal position, thus ensuring comparable expression levels of different receptor variants and in addition, eliminating potential artifacts caused by clonal differences or heterogeneous expression level. The TREx system permits inducible expression by the addition of tetracycline to the growth medium.

Transfections were performed using Fugene keeping a 1:10 ratio between POG44 (Invitrogen) and pcDNA5/FRT/TO-based vector. Transfected cells were selected by substituting zeocin with 150 μg/ml hygromycin B. Cells used in integrin structure-function analysis were, at first, transfected to stably express $uPAR^{T54A}$. Briefly, 293 Flp-In T-Rex cells were transfected using Fugene with a vector encoding for uPAR and selected with 1mg/ml G418. Clones obtained by limiting dilution where screened for uPAR expression level. Cells obtained in this way were further transfected and selected, using the Flp-In system, as described above. Plasmids for transfections were generated in the lab. Briefly cDNA from uPAR, β_1 integrin, β_3 integrin, PAI-1 and VN were cloned in pcDNA/FRT/TO

vector for flip-in cells and mutagenized with QuickChange II site directed mutagenesis protocol (Stratagene).

Expression and purification of recombinant proteins

Semi-confluent CHO-Flp-In cells, stably transfected with the specific vectors encoding for VN variants, were washed with PBS and incubated for one or two weeks in CHO protein-free medium (SIGMA Aldrich). The supernatant were collected and VN variants were purificated with nickel beads.

Adhesion assay

96 well plates were coated with purified substrates overnight at 4° (poly-D-lysine 100 μg/ml , FN 10 μg/ml , anti-$\alpha_v\beta_3$ integrin antibody 20 μg/ml, VN, VN^{RAD}, $VN^{\Delta SMB}$, $VN^{RAD\Delta SMB}$ were all coated at 5 μg/ml) and blocked for 2 hours at 37° with 2% heat-inactivated BSA in PBS. Cells were washed, harvested and counted. After 3 washes with binding buffer (DMEM supplemented with penicillin 100 U/ml, streptomycin 100 U/ml, L-glutamine 5mM, 25 mM HEPES and 0.1% BSA) equal number of cells were seeded (3 x 10^4 cells/well) and allowed to adhere for 30 minutes in presence or absence of uPA (10nM) or integrin-blocking antibodies (anti-$\alpha_v\beta_5$ P1F6, anti-$\alpha_5\beta_1$ P1D6, anti-β_1 4B4 and anti-β_1 mab13 all used at 5μg/ml). After washing, adherent cells were fixed with 4%PFA and stained with crystal violet. Cell adhesion was quantified measuring absorbance at 540 nm.

Cell Seeding for spreading and signaling assays

12 well plates (for western blot/signaling experiments) or glass-bottom plates (for imaging or time lapse experiments) were coated overnight at 4° with different substrates (poly-D-lysine 100 μg/ml , FN 10 μg/ml , α-uPAR antibody R4 20 μg/ml, anti-$\alpha_v\beta_3$ integrin antibody LM609 20 μg/ml, VN, VN^{RAD}, $VN^{\Delta SMB}$, $VN^{RAD\Delta SMB}$ were all coated at 5 μg/ml) and blocked for 2 hours at 37° with 5% heat-inactivated BSA in PBS. Detached cells were washed 3 times in binding buffer and counted. 2,5 x 10^5 cells/well for Immunoblot experiments or 2 x 10^4 cell/well for imaging and time lapse experiments were seeded and allowed to adhere for 30 minutes in presence or absence of uPA (10nM). In chase of antibody or inhibitors treatment, cells were pre-incubated with integrin-blocking antibodies (anti-$\alpha_v\beta_5$ P1F6, anti-$\alpha_5\beta_1$ P1D6, anti-β_1 4B4 and anti-β_1 mab13 all used at 10μg/ml) or inhibitors (PP1, PP2 and PP3 10 μM, UO126 20 μM and AG1478 250 nM) in suspension for 15 or 30 minutes respectively before plating. For off-plate experiments cells were seeded on BSA blocked plates for 30 minutes in presence of R4 (20 μg/ml) or VN^{RAD} and uPA (respectively 5 μg/ml and 10 nM).

Immunoblot

Eventually non adherent cells were collected. Cells were lysed in 95°C laemmli buffer (60 mM Tris-Cl pH 6.8, 2% SDS, 10% glycerol, 0.01% bromophenol blue) or in ice-cold RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, protease inhibitor cocktail [Complete-EDTA-free], 1 mM PMSF, 1 mM EDTA, 1 mM NaF, and 1 mM Na_3VO_4). Equal amount of protein were separated by

SDS-page, transferred to nitrocellulose membranes and probed as indicated in the figures. Different biological replicates were analyzed by densitometry using imageJ.

DIC microscopy

Adherent cells were fixed with 4% PFA (in PBS) for 10 minutes at room temperature. Fixed cells were washed with PBS and DIC imaging of cells was performed using an inverted microscope Olympus IX81. Cells were viewed through a high-aperture 60x objective lens (UIS2 60x TIRFM PlanApo N, NA 1.45; Olympus). Images were acquired using Hamamatsu Orca-ER digital camera with the software Metamorph 7.5.6.0. Cell area was quantified using imageJ.

Phase contrast and time lapse microscopy

Phase-contrast and time-lapse live-cell imaging was performed at 37°C, 5% CO_2 with an inverted microscope (IX80; Olympus) equipped with an incubation chamber (OKOlab) to control CO_2 and temperature. Cells were plated in serum-containing growth medium or on bottom-glass plates coated with substrates and viewed through 10X (for time-lapses), 20X or 60X (for phase contast pictures) objective lenses. The acquisition system includes a digital camera (Hamamatsu Orca-ER) and System Control Software Olympus ScanR. Adjustment of brightness/contrast, smoothening and sharpness of images were done using ImageJ 1.42q and always applied to the entire image. Cell migration speed was quantified

with ImageJ 1.42q using the plug-in "manual tracking". In each experiment, 20 randomly chosen cells were tracked and their average migration speed throughout the experiment was calculated.

Dynabeads experiments

Dynabeads were coated with PL, FN or VN^{RAD} following manufacturer instructions ($4x10^8$ beads were coated with 100μg of ligands) and blocked with 0.1% BSA. $uPAR^{T54A}$ cells were detached, washed and resuspended in binding buffer. $2x10^6$ cells were incubated with $4x10^6$ beads (cell/beads ratio 1:2) in presence of uPA (10nM) and 4B4 (10 μg/ml) for 30 minutes at 37°C in rotation. Cell attached to Dynabeads were captured with the provided magnet, washed 3 times with binding buffer and lysed in RIPA buffer.

SiRNA

Cells were grown to sub-confluency and transfected with stealth siRNA (talin: TLN1HSS110803, β_1 integrin ITGB1HSS105559, focal adhesion kinase PTK2 validated stealth duplex 1, control oligo: medium CG control stealth siRNA. Invitrogen) following manufacturer's protocol. SiRNA transfection was performed with stealth siRNA at 40 nM as final concentration. After one day, cells were retransfected as described above and let grow for one day. Cells were then plated for experiments so that the day after they will be at sub-confluency.

Statistic analysis:

For adhesion assays and western blot densitometry data were represented as mean ± standard error mean (s.e.m.) in at least 3 independent experiments. Cell spreading and cell migration experiment were represented as dot plot showing the value of every single cell quantified. Mean ± 95% confidence interval was calculated. At least 50 cells in two independent experiments were quantified.

RESULTS

uPAR-induced cell adhesion and signaling requires a direct uPAR/VN-interaction

uPAR expression has been shown to induce remarkable changes in signal transduction, cell adhesion, cell morphology and cell migration. The effects exerted by uPAR depend on the functional cross-talk with transmembrane receptors (receptor tyrosine kinase, G protein coupled receptors and integrins) and the interaction with its natural ligands, uPA and VN. In fact uPAR directly interacts with the extracellular matrix protein VN and uPAR-mediated VN adhesion has been reported to be sufficient and indispensable to increase cell spreading and migration in HEK 293 cells (Madsen et al., 2007).

To investigate the molecular mechanism of adhesion-induced outside-in signaling we exploited 293 cells expressing wild-type uPAR ($uPAR^{WT}$) and the T54A single alanine substitution variant ($uPAR^{T54A}$) in which VN-binding activity is conditional, being almost entirely dependent upon concomitant binding of the canonical uPAR-ligand uPA (Madsen et al., 2007).

The adhesive properties of these cells were evaluated on different ECM components in presence or absence of uPA. uPAR expression and uPA treatment did not alter cell adhesion in FN (Fig. 1a). On the contrary, when cells were seeded on VN, uPAR expression strongly increased cell adhesion to this ECM component. The expression of the $uPAR^{T54A}$ mutant enhanced VN adhesion when cells were treated with uPA, while it did not modify cell adhesion to VN in the basal condition (Fig. 1b). uPA treatment on $uPAR^{WT}$ expressing cells had little effect on VN adhesion.

Fig. 1a **Fig. 1b**

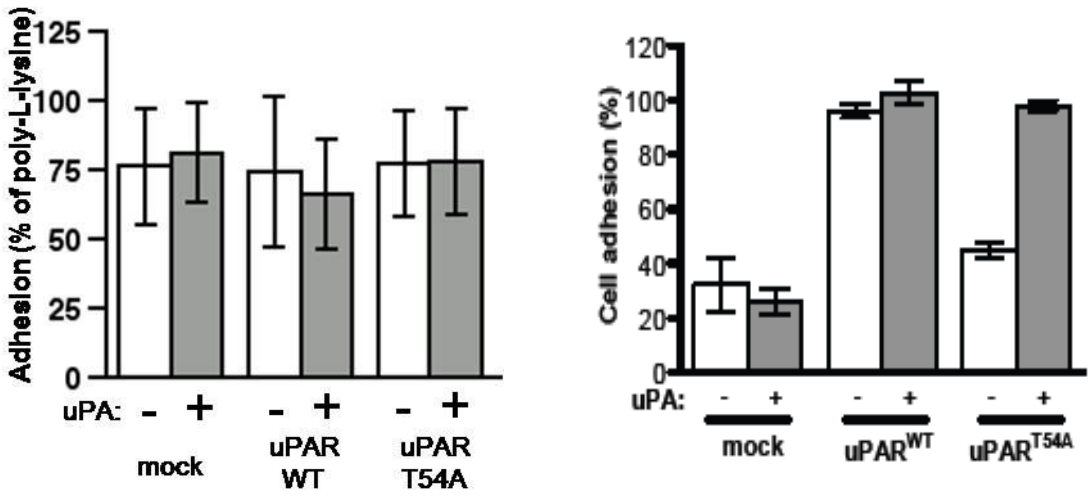


Figure 1 uPAR induces cell adhesion to vitronectin. Cell-adhesion assay: Mock, $uPAR^{wt}$ and $uPAR^{T54A}$ transfected 293 cells were plated for 30 minutes on FN (**fig 1a** 10 μg/ml) VN (**fig 1b** 5 μg/ml) with or without uPA (10nM). Adherent cells were fixed and stained with crystal violet. Cell adhesion to poly-D-lysine was set as 100% for each cell line. Data are expressed as mean ± s.e.m., n=3.

These data show that uPAR increases cell adhesion to VN without altering cell adhesion to other ECM components. Moreover, uPA treatment rescues the adhesive capability of T54A mutant, most probably by promoting a binding competent conformation.

The increased adhesion to VN is paralleled by changes in cell morphology and in signal transduction. To monitor the morphology changes upon uPAR-VN interaction, we quantified the area of individual cells through DIC microscopy and image analysis. When grown under serum containing conditions the expression of $uPAR^{WT}$, but not $uPAR^{T54A}$, results in increased cell spreading (~2.5-fold) as compared to mock-transfected cells (Fig. 2). Treatment with uPA rapidly induces spreading of $uPAR^{T54A}$ cells to levels comparable with those observed in $uPAR^{WT}$ cells, but has no or little effect on cell spreading in mock and $uPAR^{WT}$ transfected cells.

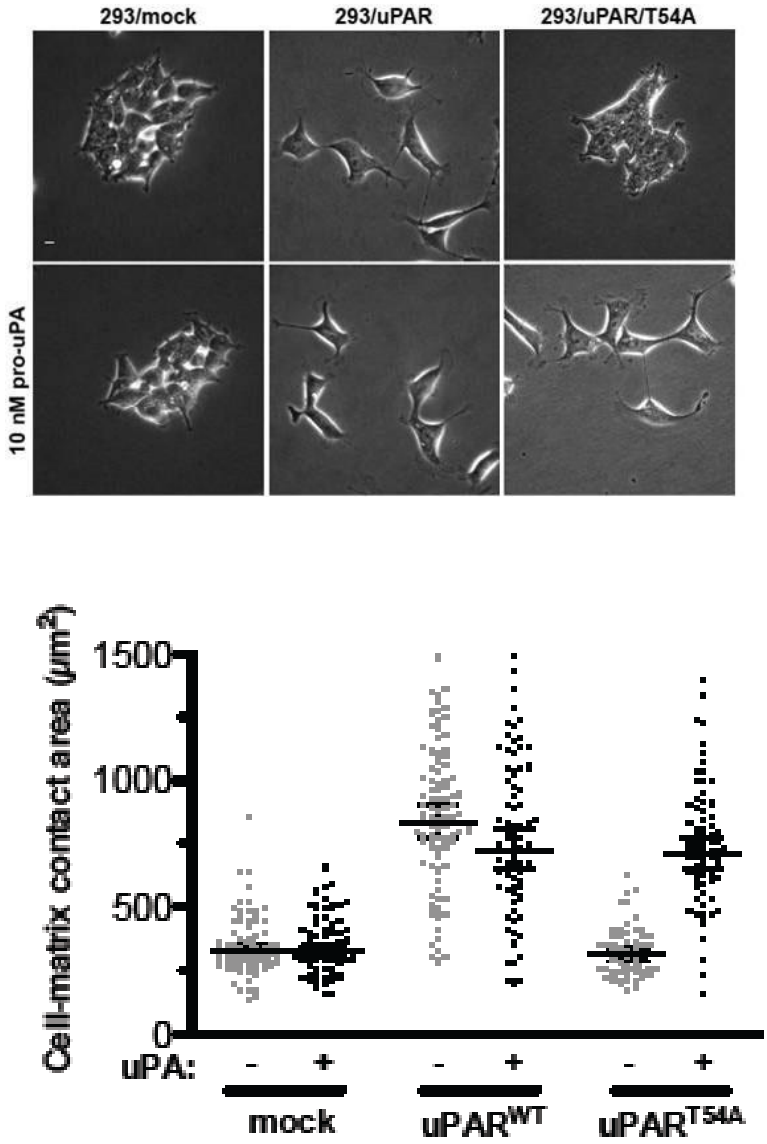


Figure 2 Increased cell-matrix contact area upon uPAR mediated VN adhesion. Quantification of cell-matrix contact area: Cells were grown overnight and stimulated for 30 minutes with uPA (10 nM). After fixation, DIC images were taken and cell areas were quantified using ImageJ software. Data are mean ± 95% confidence interval, n=50, two independent experiments. Every dot represents the area of one single cell. Representative images taken with phase contrast microscopy are shown.

The effect of uPA treatment on uPAR^{T54A} is documented in Fig.3, showing sequential images taken from a time lapse recording. uPA treatment (time 0) induced deep changes in cell morphology characterized by marked lamellipodia extensions, increase in cell spreading and the acquisition of a motile phenotype. The effects on cell morphology were visible already after 10 minutes of uPA stimulation.

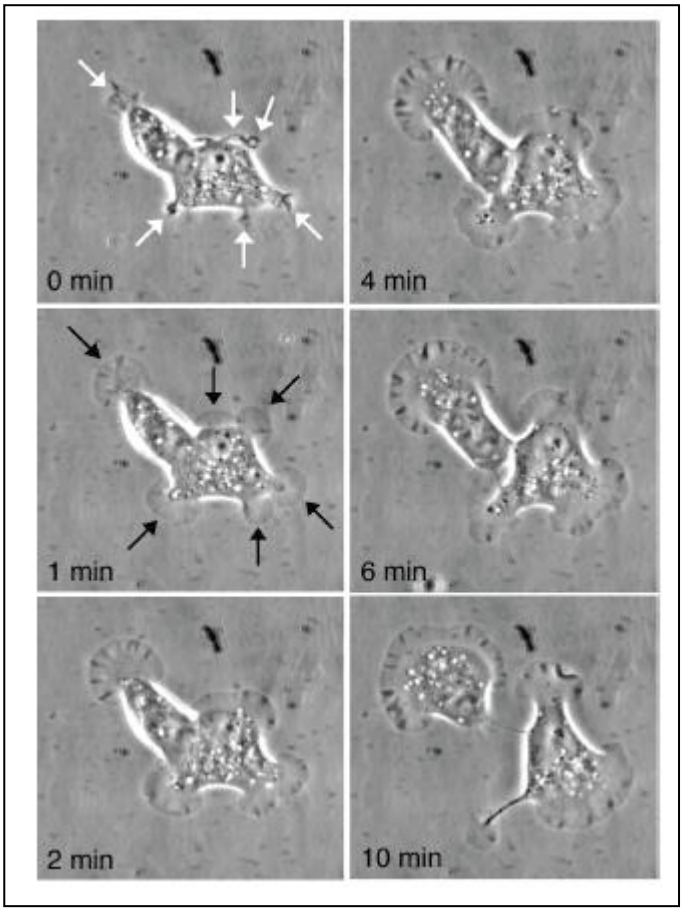


Figure 3 Rescuing VN binding capability of uPAR^{T54A} cells induces changes in cell morphology. Representative pictures taken from a time lapse of cells grown in serum containing medium are shown. At time 0 uPAR-VN interaction is induced with uPA (10nM), producing already after 1 minute effects on cell morphology.

uPAR expression is reported to trigger the activation of pro-migratory and proliferative signaling pathways. Thus, the phosphorylation of ERK1/2 (involved in proliferation) and p130Cas (involved in cell spreading and migration) was assayed through immune blotting and quantified with densitometry. Serum starved uPAR^{WT} cells displayed higher levels of p130Cas substrate domain (SD) phosphorylation and ERK1/2

phosphorylation, compared to mock transfected cells (fig 4). uPA treatment on mock or uPAR^{WT} expressing cells had little or no effect on signal transduction. On the other hand, in cells expressing the uPAR^{T54A} the increase in p130Cas SD phosphorylation and MAPK-activation was strictly uPA-dependent documenting the conditional properties of this receptor (Fig 4).

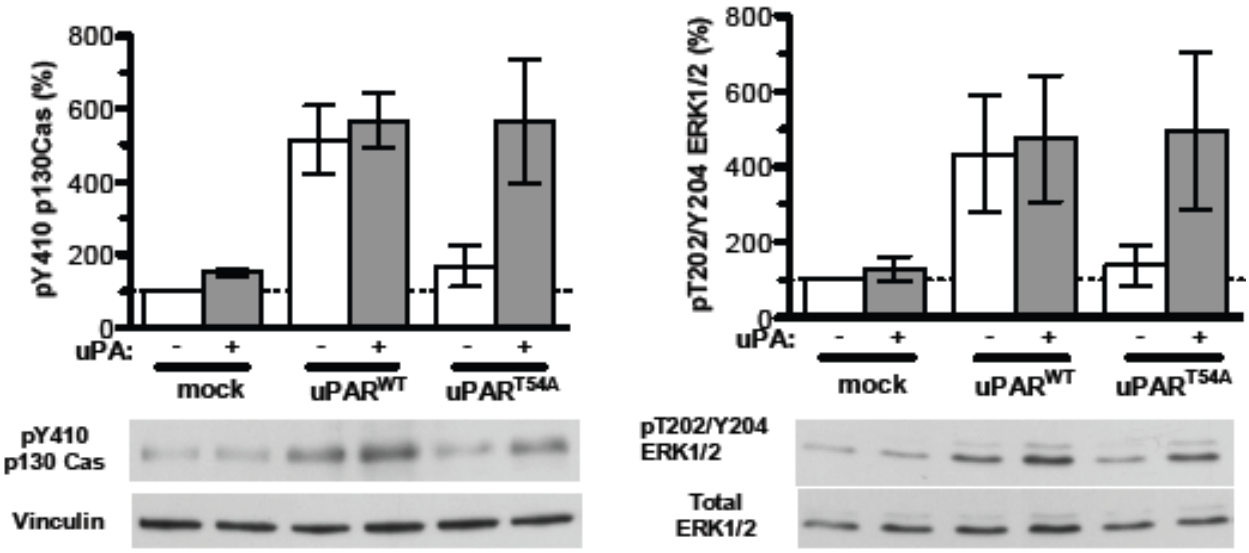


Figure 4 Immunoblot analysis of p130Cas SD in Y410 and ERK1/2 in T202/Y204 phosphorylation upon uPAR-VN interaction. Western blot and densitometric analysis: Cells were serum starved for 4 hours and stimulated, where indicated, with uPA (10 nM) for 30 minutes prior to lysis. p130Cas and ERK1/2 phosphorylation was assayed with immunoblot and quantified with densitometry. Ratio of mock untreated cells was set as 100%. Data are means ± s.e.m., n=3. Representative western blot are shown.

Taken together these data show that uPAR is an adhesion receptor specific for VN and that uPAR-mediated cell adhesion to VN is paralleled by changes in signal transduction and in cell morphology. The activity of uPAR in inducing these changes is strictly related to its adhesive function, as the expression of the T54A mutant (defective in VN adhesion without uPA treatment) did not cause any visible effect. Consistently uPA treatment rescues the adhesive capability of this mutant triggering cell spreading and signal transduction. uPA did not produce any affect on uPAR^{WT} and mock transfected cells,

demonstrating the specificity of this system. Indeed uPAR WT cells strongly adhere to VN and display activated signaling even without uPA.

The conditional properties of T54A receptor were further used to study the nature of the signaling deriving from uPAR-VN interaction. uPAR is a signaling incompetent molecule by itself, lacking a cytoplasmic tail, and the functional cross-talk between uPAR and integrins frequently recurs in literature. The morphology changes observed together with the phosphorylation on p130Cas could indicate the involvement of integrin receptors into uPAR-VN signaling.

To test this possibility we perform siRNA experiment in $uPAR^{T54A}$ cells targeting different proteins involved in integrin signaling. We decided to knock-down β_1-integrin that is the most expressed integrin expressed in 293 cells, talin that plays a crucial role in the integrin activation process and focal adhesion kinase (FAK) that, together with Src, plays an important role in integrin out-side-in signaling. Immunoblot analysis revealed that the three siRNAs specifically down-regulate the level of their target protein, without interfere with other proteins in unspecific ways (fig 5a).

We investigated the effect of these siRNA on uPAR-VN signaling by stimulating $uPAR^{T54A}$ with uPA and analyzing the level of p130Cas phosphorylation (Fig 5b). Immunoblots revealed that the down-regulation of β_1-integrin, talin and FAK impaired p130Cas phosphorylation upon uPA treatment, indicating their proficiency in inhibiting the signaling triggered by uPAR-mediated VN adhesion.

Fig 5a

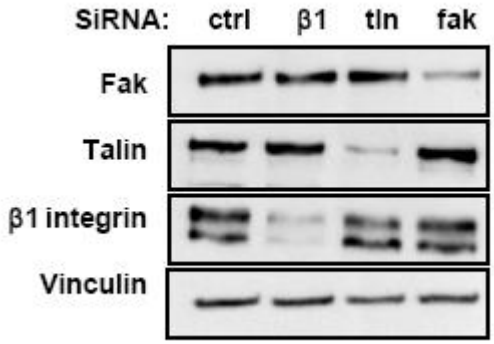


Fig 5b

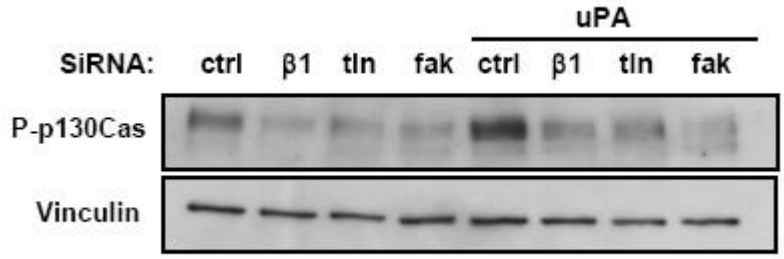


Figure 5 Down-regulation of β_1-integrin, talin and focal adhesion kinase impairs uPAR-VN signaling. SiRNA and western blot analysis: **5a** 293 $uPAR^{T54A}$ were subjected to 2 cycles of siRNA treatment: ctrl (control siRNA), β_1 (β_1-integrin siRNA), tln (talin siRNA), FAK (focal adhesion kinase siRNA). Cell lysates were analyzed by immunoblot to show the specific down regulation of the target proteins. **5b** Interfered cells were serum starved for 4 hours and stimulated with uPA (10nM) for 30 minutes where indicated. p130Cas was analyzed through immune blot. Representative western blots are shown.

This data demonstrate that important components of the integrin-signaling machinery like β_1-integrin, focal adhesion kinase and talin are crucial mediators of the uPAR-VN signaling. This indicates uPAR-VN interaction induces cell adhesion to vitronectin and triggers an integrin-dependent signaling characterized by p130Cas and ERK1/2 phosphorylation, changes in cell morphology and enhanced cell spreading. Thus, the role of integrins will be further analyzed.

uPAR-induced cell spreading, cell migration and signaling on VN are RGD independent

An attractive explanation for the signaling activity of uPAR on VN-containing matrices is that the uPAR-driven increase in cell adhesion brings VN-binding integrins to engage the matrix thus potentiating outside-in signaling from these receptors. To address this possibility directly we utilized recombinant VN-variants where we had specifically disrupted the integrin-binding site by mutation of the RGD motif into RAD (VN^{RAD}), uPAR-binding by deletion of the SMB-domain ($VN^{\Delta SMB}$) or both ($VN^{RAD\Delta SMB}$). It is well established that the RGD-motif represents the key integrin binding site in VN (Xiong et al., 2002). The uPAR binding site is located in the SMB-domain that is functionally and physically separated from the integrin binding motif (Fig. 6) (Madsen et al., 2007; Okumura et al., 2002).

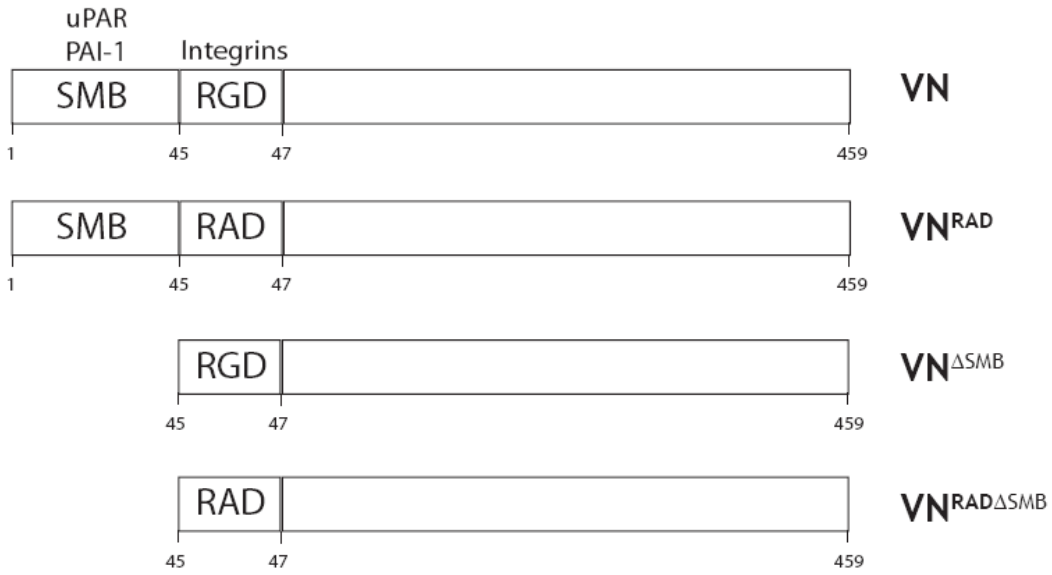


Figure 6 Schematic representation of VN variants: SMB domain (somatomedin b domain) in the N-terminal represents uPAR and PAI-1 binding sites. RGD motif represents the integrin binding site.

The properties of the recombinant VN-molecules were first confirmed in adhesion assays using $uPAR^{T54A}$ cells in the absence or presence of uPA to compare cell adhesion mediated by integrins alone or by integrins and uPAR together, respectively (Fig 7).

When the T54A mutant is in a binding incompetent conformation (without uPA), cell adhesion is entirely supported by VN-integrins. Indeed it was not perturbed by SMB-domain deletion (on $VN^{\Delta SMB}$), as the SMB domain does not contain integrin binding sites. On the contrary, it was strongly dependent on RGD motif integrity (on VN^{RAD}). When T54A receptor is in its binding competent conformation (with uPA), cell adhesion was strongly increased on the VN variants containing the SMB domain (VN and VN^{RAD}). Importantly uPAR-mediated VN adhesion is integrin-independent, as the disruption of the integrin specific RGD motif did not impair uPAR-mediated VN adhesion (on VN^{RAD} with uPA). No residual adhesion is detected when both the RGD-motif and the uPAR/VN-interaction are impaired contemporarily ($VN^{RAD\Delta SMB}$). These data show that the use of the 293^{T54A} cells in combination with the different recombinant VN-variants allows for the functional dissection of the contribution of uPAR and integrins in VN-induced cell adhesion, spreading and signaling.

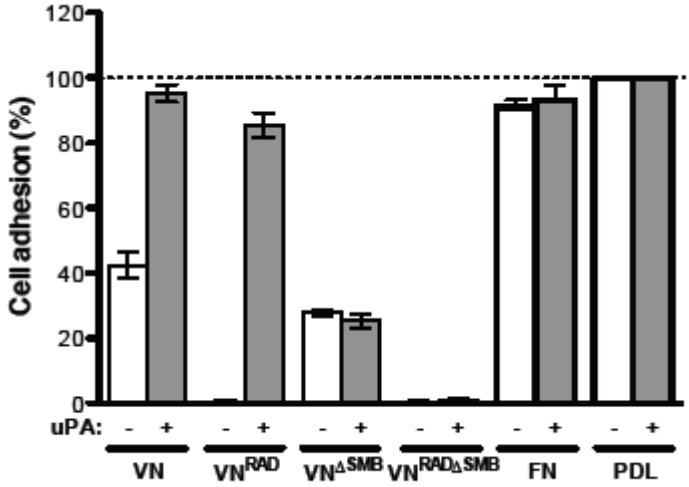


Figure 7 Effect of vitronectin variants on uPAR and integrin dependent cell adhesion. Cell-adhesion assay: uPAR^{T54A} cells were plated on vitronectin variants (5 μg/ml) or on fibronectin (10 μg/ml) for 30 minutes, uPA was added where indicated. Cell were fixed and stained with crystal violet. Cell adhesion was quantified and expressed as percentage of Poly-D-lysine adhesion. Data are expressed as mean ± s.e.m, n=3.

To address the role integrin-matrix interaction in uPAR-induced cell spreading we seeded uPAR^{T54A} cells in the absence or presence of uPA on VN, VN^{RAD}, FN and poly-D-Lysine (Fig. 8) and assayed cell spreading after 30 minutes incubation at 37˚C. Remarkably, cell spreading downstream of uPAR-mediated adhesion to VN was entirely comparable on VN and VN^{RAD}, suggesting that the integrin binding site in VN is of no or little importance in the process. The degree of cell spreading induced by the uPAR/VN-interactions is similar to the one mediated by canonical integrin-dependent cell adhesion to FN. In the absence of the uPAR-binding to VN (i.e. in the absence of uPA), cell spreading on VN is reduced and comparable to that observed on poly-D-lysine and in mock-transfected cells on serum coated surfaces. The presence or absence of uPA had little or no effect on cell spreading on FN and PLD supporting the notion that the activity of this molecule is specifically related to its ability to promote uPAR binding to VN.

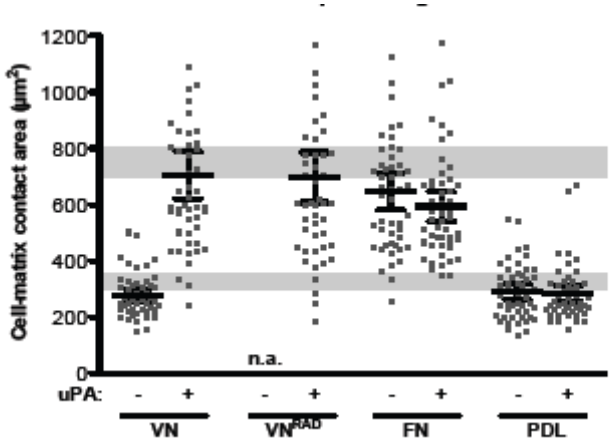


Figure 8 uPAR-induced cell spreading does not require vitronectin integrin engagement. Quantification of cell-matrix contact area: Cells were plated on VN variants (10 µg/ml), fibronectin (10 µg/ml) or poly-D-lysine (100 µg/ml) with or without uPA (10 nM) for 30 minutes at 37°C. Quantification of cell-matrix contact area of DIC images was performed. Upper Grey area represents the range of fully spread cells, lower grey area is related to not spread cells. These ranges are based on confidence intervals of Fig. 2. Absent cell adhesion prevented basal VN^{RAD} cell spreading quantification. Data are mean ± 95% c.i., n=50 in two independent experiments.

Similar data were obtained when adhesion-induced signal transduction was analyzed in cells seeded on the different substrates: uPAR-mediated cell adhesion to VN induced p130Cas SD phosphorylation and MAPK-activation (Fig. 9) to levels that are comparable with those observed during integrin mediated cell adhesion to FN. Importantly this signaling was insensitive to the integrity of the integrin binding site in VN, as the level of p130Cas and ERK1/2 phosphorylation in cells adhering through uPAR to VN^{RAD} (VN^{RAD} treated with uPA) was comparable to the one observed on VN. uPA treatment did not induce signal transduction on substrates in which the SMB domain was depleted (VN^{SMB} and $VN^{RAD\Delta SMB}$), consistently with the lacking of the uPAR binding region on this VN variants. Thus, the interaction between uPAR and VN is strictly required to induce changes in signal transduction, while integrin binding to the VN RGD motif is dispensable.

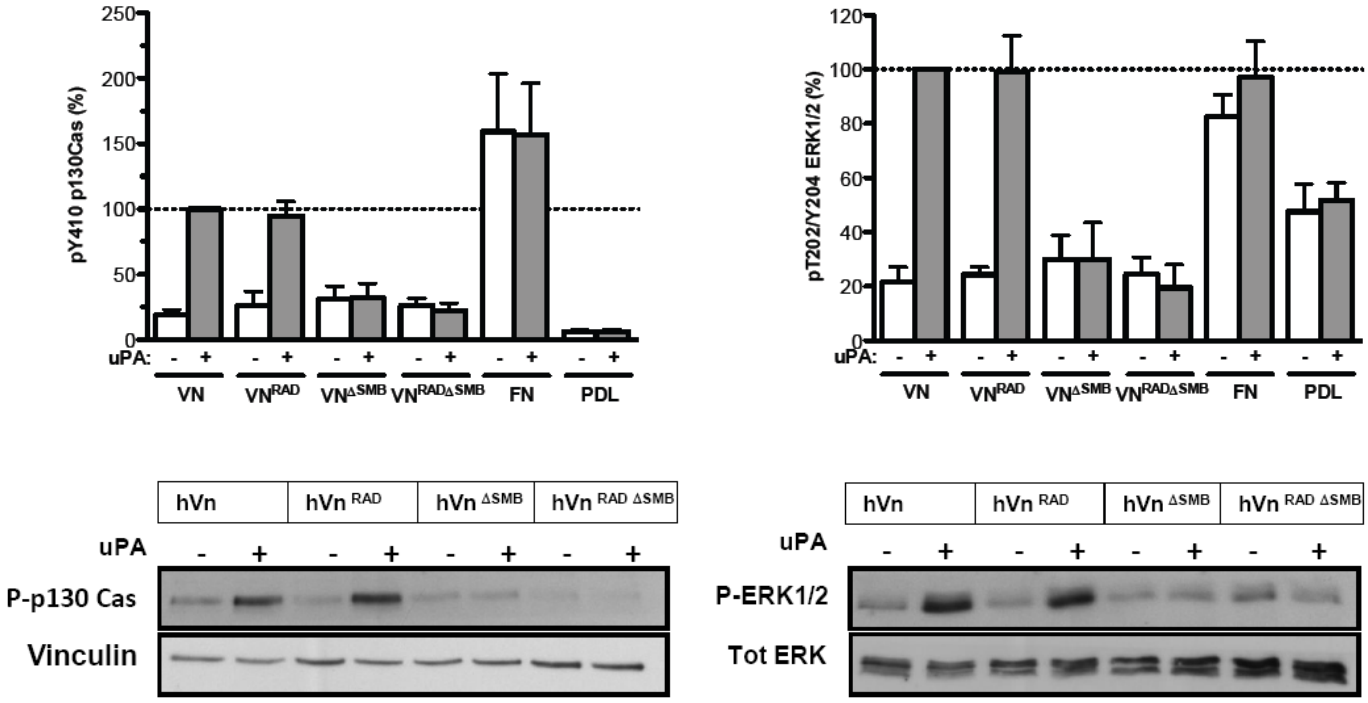


Figure 9 Analysis of Y410 p130Cas and T202/y203 ERK1/2 phosphorylation on VN variants. Western blot and densitometric analysis: Cells were seeded on VN variants (10 µg/ml) in presence or absence of uPA (10nM) for 30 minutes at 37°C. P130Cas and ERK1/2 phosphorylation was assayed with immunoblot and quantified. Ratio of cells plated on VN and stimulated with uPA was set as 100%. Data are represented as mean ± s.e.m., n=3

The uPAR/VN interaction induces ligand-independent signaling through β_1 integrin

The above findings document that uPAR-mediated VN adhesion induces a signaling cascade similar to integrin outside-in signaling through a mechanism that appear to occur in the absence of integrin binding sites in the matrix. Down-regulation of β_1 integrin through siRNA technology results in impaired uPAR signaling to p130Cas. Thus the role of integrins in uPAR-VN signaling has been investigated by testing series of inhibitory anti-integrin antibodies for their ability to interfere with integrin-mediated cell adhesion to VN and FN as well as their effects on uPAR-induced cell adhesion, spreading and p130Cas SD phosphorylation on VN^{RAD}, using $uPAR^{T54A}$ cells as model. The analysis of 293 cells transcriptome revealed high levels of β_1 integrin transcript, indicating a major role of this subunit in integrin-mediated cell adhesion and signaling. Furthermore, the modest level of α_V and β_5 subunits and the absence of β_3 integrin transcript could indicate that the endogenous VN-receptor in 293 cells is $\alpha_V\beta_5$-integrin. Indeed the weak integrin-mediated VN adhesion of 293 cells is mediated by the $\alpha_V\beta_5$-integrin as evidenced by the specific inhibitory effect of the function-blocking antibody P1F6. Importantly the blocking of β_1 integrin through functional inhibitory antibodies (4B4 and mAb13) did not impair VN adhesion, showing that this integrin is not involved in cell-adhesion to this ECM component. On the other hand a $\alpha_5\beta_1$ function-blocking antibody, P1D6, as well as two different allosteric inhibitory β_1-antibodies, 4B4 and mAb13, specifically impaired cell adhesion to FN. None of the antibodies had any effect on uPAR-mediated cell adhesion to VN^{RAD} ($uPAR^{T54A}$ cells with uPA) in accordance with cell binding to this substrate being mediated exclusively by the direct uPAR/VN-interaction (Fig. 10).

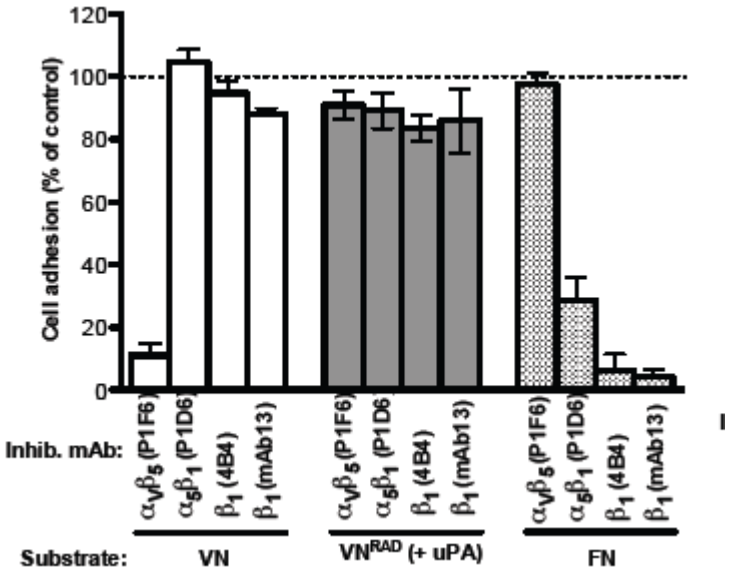


Figure 10 Adhesive properties of 293 uPAR^{T54A} cells. Cell adhesion assay: Cells were plated on the indicated substrates (VN and VN^{RAD} 5 μg/ml, FN 10 μg/ml) for 30 minutes in presence of integrin blocking antibodies (5 μg/ml) and uPA (10 nM) where indicated. Cell adhesion was measured and expressed as percentage of the untreated controls. Data are means ± s.e.m., n=3.

We then tested the effect of integrin blockage on uPAR/VN mediated signaling and cell spreading by plating uPAR^{T54A} cells on VN or VN^{RAD} in presence of integrin inhibitory antibodies. When the same antibodies, used in adhesion assays, were tested individually for their inhibitory effect on uPAR/VN-induced cell spreading on VN (Fig. 12) and p130Cas SD phosphorylation (Fig. 11) only very modest effects were observed. However, the combined inactivation of $\alpha_V\beta_5$ and β_1 resulted in a virtually complete inhibition of both cell spreading and p130Cas SD phosphorylation. When cells were seeded on VN^{RAD} the allosteric interference with β_1-function alone was sufficient to attain almost complete inhibition in terms of cell spreading and p130Cas phosphorylation (Fig. 11 and Fig 12). The blocking of either $\alpha_V\beta_5$ function had no or only marginal effect. The data thus show that both $\alpha_V\beta_5$ and β_1-integrins are capable of transducing the signal

triggered by the uPAR/VN-interaction. On integrin permissive VN the individual inhibition of these integrins has limited effect while the combined inhibitions abolish the biological effects documenting an evident functional redundancy between these two receptors in the transmission of the uPAR/VN-signal. On VN^{RAD}, where $\alpha_V\beta_5$-binding is blunted, this redundancy is lost and almost complete inhibition can be attained by allosteric interference with β_1-function alone even if this integrin is not involved in cell adhesion to VN (Fig. 10).

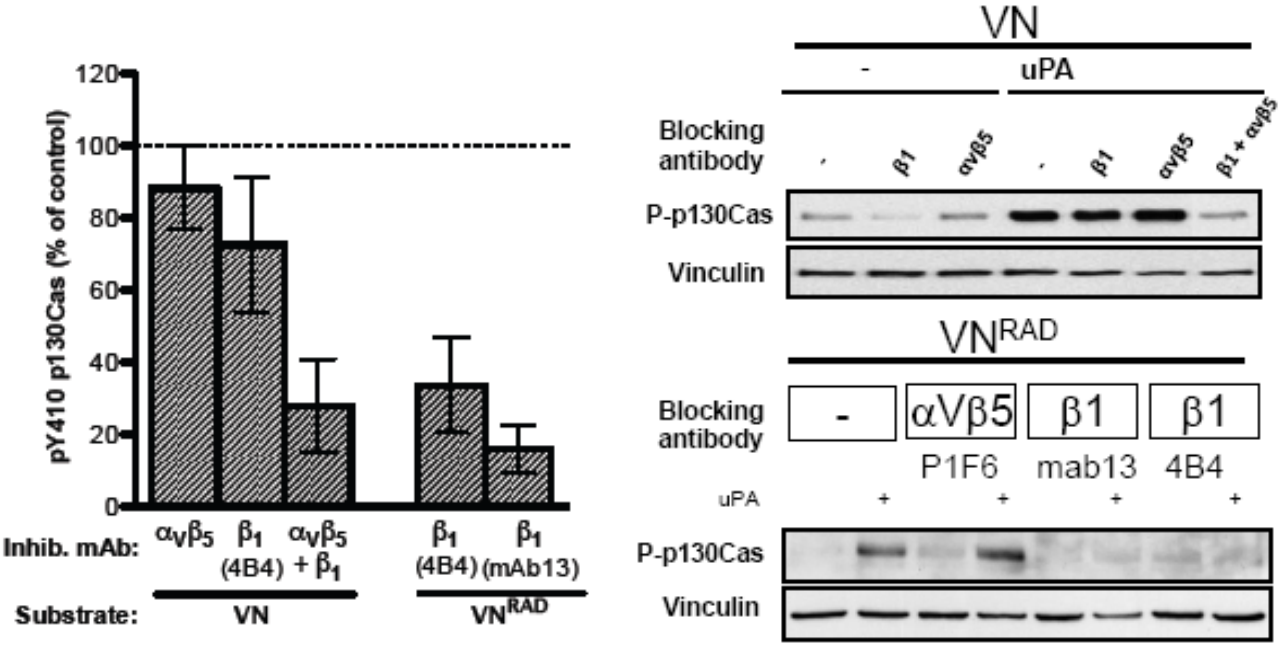


Figure 11 β_1 integrin active state is required for uPAR-induced p130Cas phosphorylation. Western blot and densitometric analysis: $uPAR^{T54A}$ cells were pre-incubated with integrin-blocking antibodies (10 μg/ml) for 15 minutes and plated on either VN or VN^{RAD} (10 μg/ml) in presence of uPA (10 nM). After 30 minutes p130Cas phosphorylation in Y410 was assayed and expressed as percentage of controls without inhibitory antibodies (set as 100%) on either VN or VN^{RAD}. Data are represented as mean ± s.e.m., n=3. Representative western blot are shown.

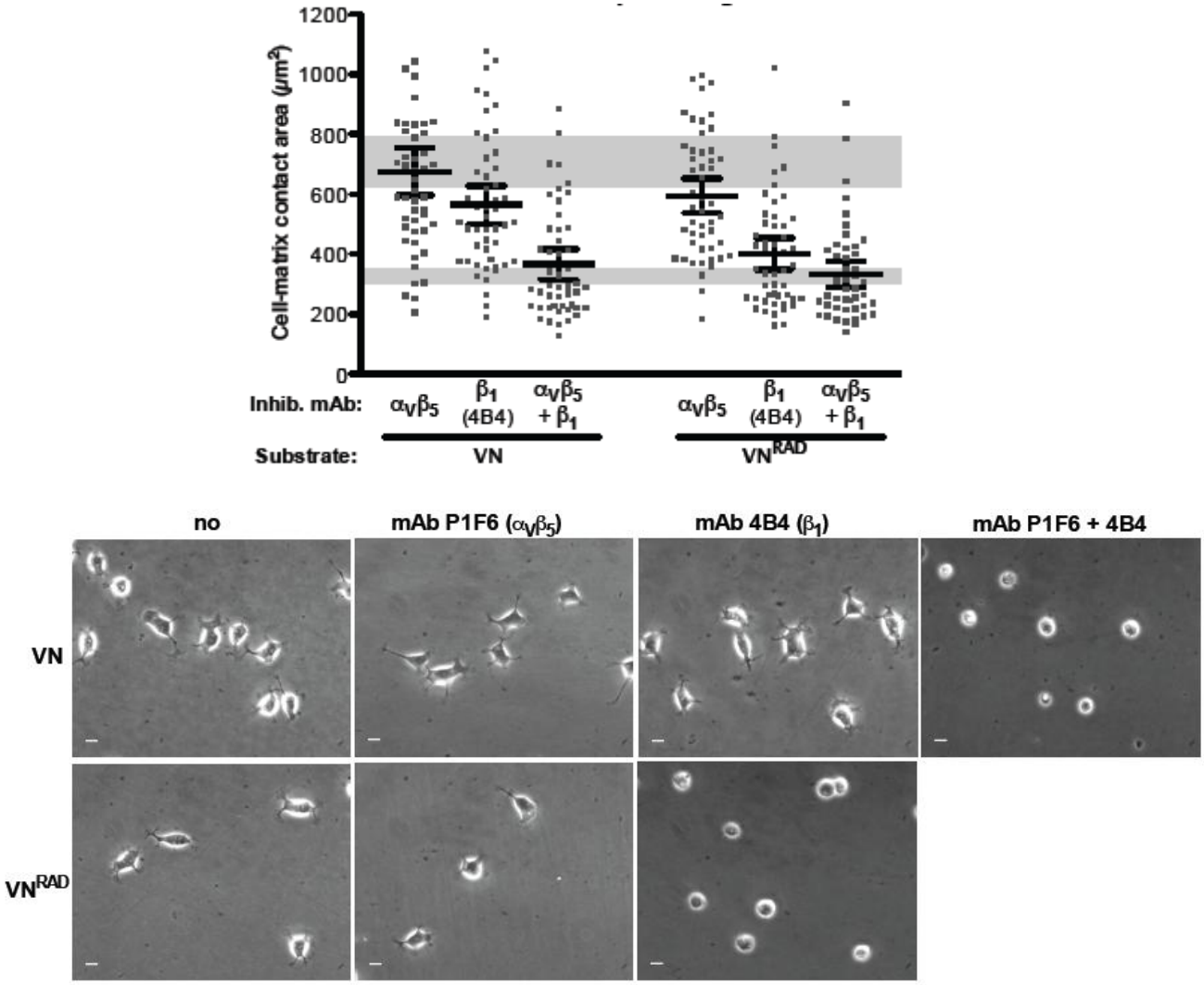


Figure 12 uPAR-induced cell spreading requires β_1 integrin active state but not ligand binding. Quantification of cell-matrix contact area: After 15 minutes pre-incubation with the indicated integrin-blocking antibodies (10 μg/ml), $uPAR^{T54A}$ cells were plated on either VN or VN^{RAD} (10μg/ml) in presence of uPA (10 nM). After 30 minutes DIC and phase contrast images were respectively taken. Cell area was quantified from DIC images and data are represented as mean 95% c.i., n=50 in two independent experiments. Upper and lower grey area represents respectively the range of fully or not spread cells, based on the confidence intervals retrieved in Fig. 2. Representative pictures of the cells are shown.

The data thus documents two parallel pathways for the transduction of uPAR/VN-signaling in 293 cells. The first of these pathways acts by ligand-dependent transactivation of the $\alpha_V\beta_5$ integrin induced by the uPAR/VN-interaction. The second, and predominant, signaling pathway is mediated almost exclusively by a β_1-integrin and apparently occurs

independently of integrin binding to the matrix. We will refer to this novel mechanism of signal transduction as “ligand-independent integrin signaling”.

Ligand-independent integrin signaling poses no identifiable constraints to the receptor inducing cell adhesion with respect to ternary-structure, ligand type or means of membrane anchorage

According to a popular paradigm for integrin-mediated uPAR signaling most of the above findings could conveniently be explained by a paradigm in which direct lateral interaction between uPAR and the integrin(s) results in conformational changes in the receptor and induce downstream signaling. However, the fundament of this paradigm, i.e. the existence of functionally important, specific and direct molecular contacts between uPAR and the signaling integrin, has been strongly questioned by exhaustive mutagenesis and genetic complementation assays demonstrating that the *only* functionally relevant direct uPAR-interaction in the process is with VN (Madsen et al., 2007).

To conclusively substantiate the notion that direct interactions between the two receptors are entirely dispensable in the process of uPAR-mediated β_1-transduced adhesion signaling and cell spreading, we also analyzed the biological properties of an artificial VN-receptor composed of the plasminogen activator inhibitor-1 (PAI-1) linked to the membrane by a GPI-anchor (PAI-1_{GPI}, (Madsen et al., 2007)). This artificial receptor shares no sequence or structure homology with uPAR but still induces strong adhesion to VN^{RAD} (Fig. 13). Conversely a VN-binding deficient variant of this receptor (103/112/125A, PAI-1_{GPI}^{VN-}) failed to induce cell adhesion to VN^{RAD} (Fig. 13). The

treatment with 4B4 did not affect cell adhesion to VN^{RAD}, being exclusively mediated by PAI-1/VN interaction (Fig. 13).

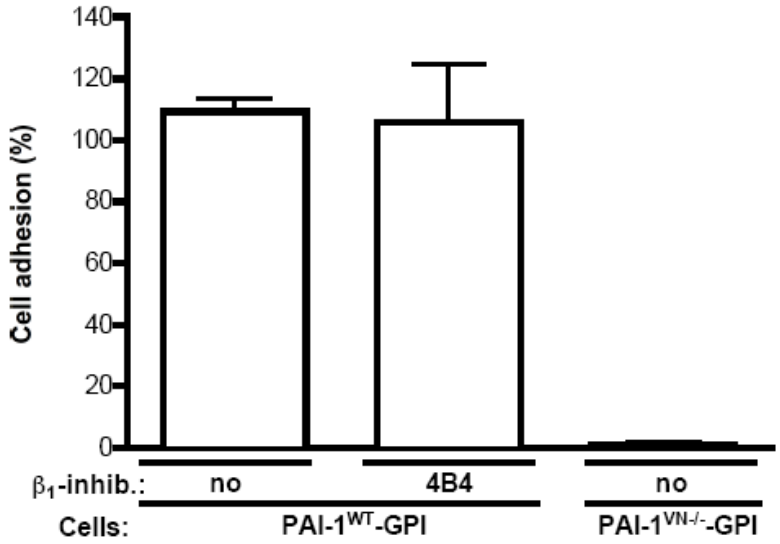


Figure 13 $PAI\text{-}1_{GPI}$ expressing cells adhere to VN^{RAD}. Cell-adhesion assay: cell expressing $PAI\text{-}1_{GPI}$ or $PAI\text{-}1_{GPI}{}^{VN-}$ were plated on VN^{RAD} (5 μg/ml) for 30 minutes. 4B4 antibody (5 μg/ml) was added where indicated. Cell were fixed and stained with crystal violet. Cell adhesion was quantified and expressed as percentage of Poly-D-lysine adhesion. Data are expressed as mean ± s.e.m, n=3.

Thus, we tested if the PAI-1-induced VN adhesion can recapitulate the same effects of uPAR-VN interaction. We found that the $PAI\text{-}1_{GPI}$ receptor induces robust p130Cas phosphorylation (Fig. 14) and cell spreading (Fig 15) when cells were seeded on VN^{RAD}. The VN-binding deficient receptor ($PAI\text{-}1_{GPI}{}^{VN-}$) failed to induce signaling to p130Cas, indicating that the ability of $PAI\text{-}1_{GPI}$ to interact with the VN-matrix is required in this process (Fig. 14). $PAI\text{-}1_{GPI}$ expression did not alter the canonical outside-in signaling to p130Cas upon plating the cells on FN and did not induce signaling on a non-integrin

substrate as PL (Fig. 14). Importantly, signaling and cell spreading downstream of PAI-1_{GPI} was β_1-dependent since it was strongly blunted by the 4B4 antibody (Fig. 14 and Fig. 15). Thus, PAI-1_{GPI} recapitulates the effects on signaling and cell spreading triggered by uPAR. This could indicate that cell adhesion molecules, that induce VN adhesion, can trigger ligand-independent integrin signaling without directly interacting with integrins.

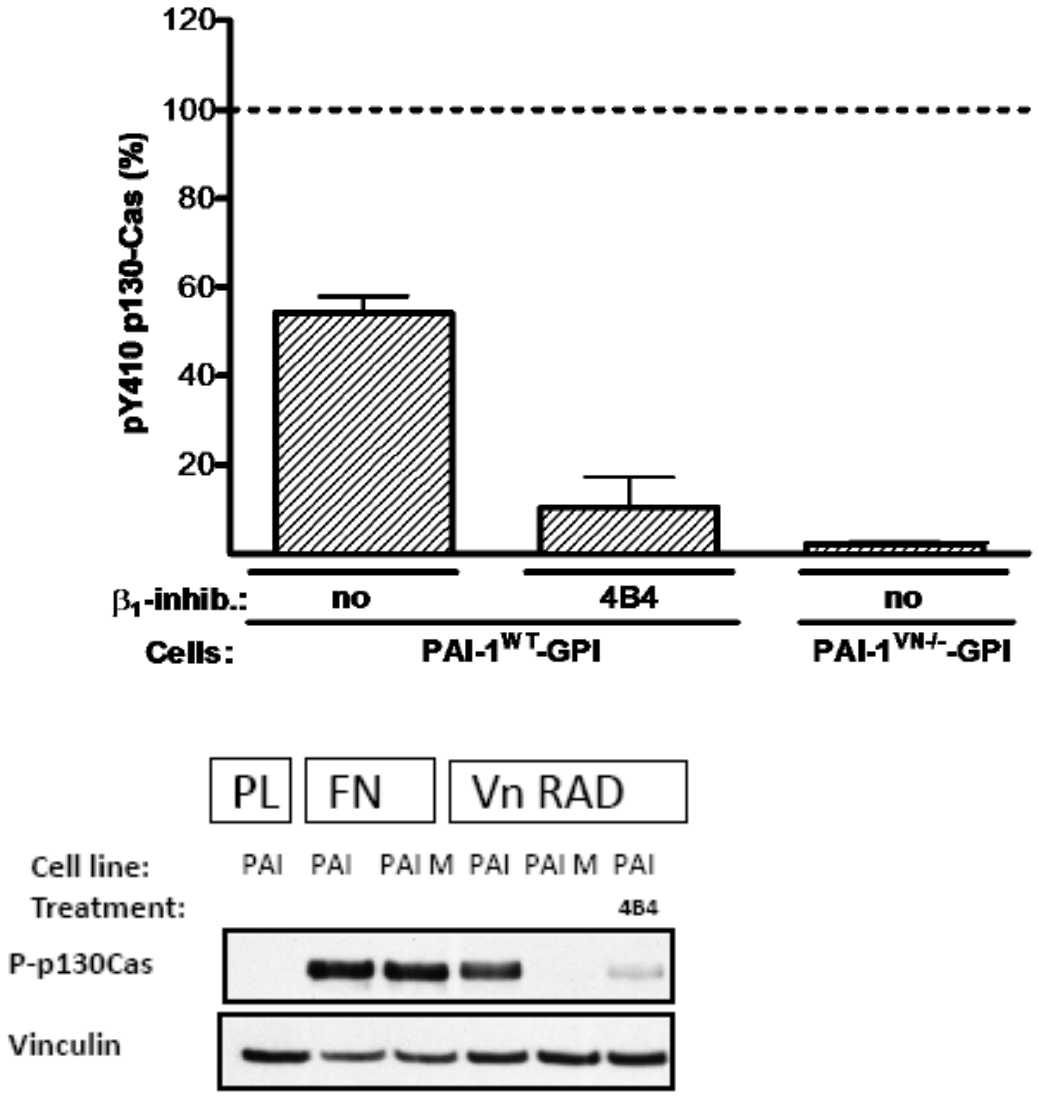


Figure 14 PAI-1_{GPI} mediated cell adhesion to VN^{RAD} induces intracellular signaling through β_1 integrin. Western blot and densitometric analysis: cell expressing PAI-1_{GPI} or PAI-1_{GPI}^{VN-} were pre-incubated with 4B4 antibody (10 μg/ml, 15 minutes) where indicated and plated on VN^{RAD} (10 μg/ml), PL (100 μg/ml) or VN^{RAD} (10 μg/ml) for 30 minutes. p130Cas phosphorylation was quantified from WB and expressed as percentage of phosphorylation of 293 $uPAR^{T54A}$ on VN^{RAD} with uPA, data are means ± s.e.m., n=3. Representative western blots are shown.

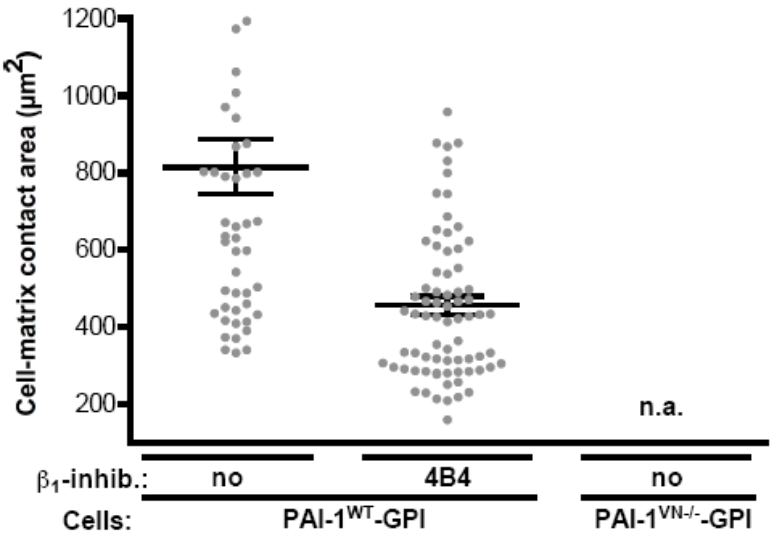


Figure 15 PAI-1_{GPI} mediated cell adhesion to VN^{RAD} induces cell spreading through β_1 integrin. Quantification of cell-matrix contact area: cell expressing PAI-1_{GPI} were pre-incubated with 4B4 antibody (10 µg/ml, 15 minutes) where indicated and plated on VN^{RAD} (10 µg/ml), for 30 minutes Cell area was quantified from DIC images and data are represented as mean 95% c.i., n=50 in two independent experiments. Upper and lower grey area represents respectively the range of fully or not spread cells, based on the confidence intervals retrieved in Fig. 2.

The fact that both uPAR and PAI-1_{GPI} share the same extracellular matrix ligand (VN) and have overlapping binding sites in this molecule could suggest that the observed signaling might be specific for this ECM component. To determine if the same or similar signaling can be triggered by *any* strong adhesion substrate we seeded uPAR-expressing cells on surfaces coated with an anti-uPAR mAb (R4) and we measured cell spreading and signal transduction in the presence and absence of the inhibitory $\beta_1$antibody 4B4. Consistent with a similar, or identical, mechanism of signal transduction, cell adhesion generated by uPAR-R4 interaction triggered strong p130Cas phosphorylation (Fig. 17) and cell spreading (Fig 16). Importantly, both cell spreading and p130Cas SD phosphorylation was fully inhibited by the 4B4 antibody (Fig. 16 and Fig. 17). R4 antibody does not

contain cryptic integrin binding sites and 4B4 antibody did not affect uPAR-mediated cell adhesion on this substrate (data not shown).

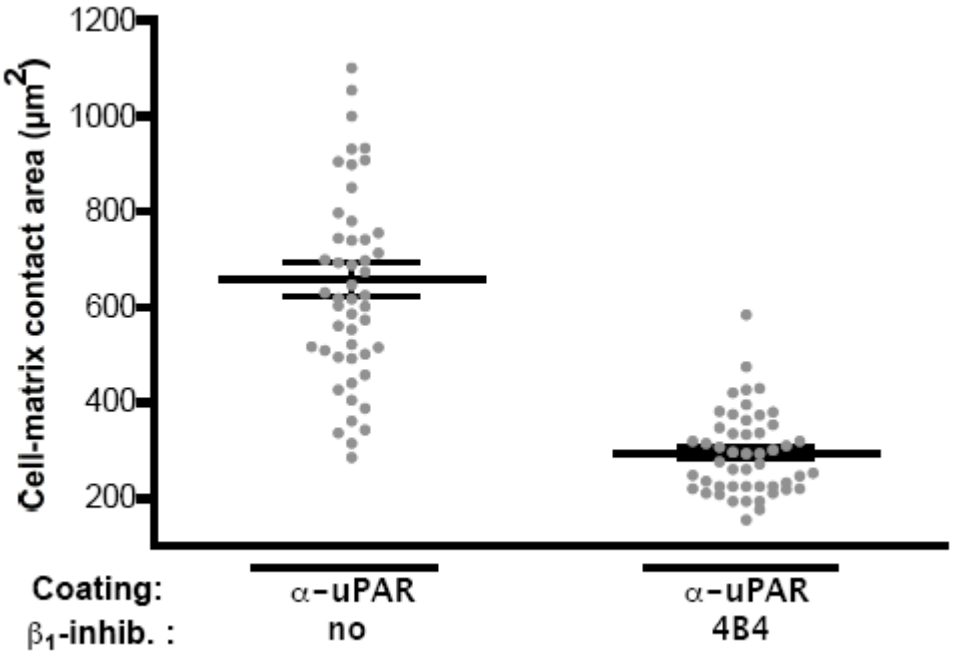


Figure 16 uPAR-R4 interaction triggers β_1 integrin dependent cell spreading. Quantification of cell-matrix contact area: $uPAR^{T54A}$ cells were pre-incubated with antibody 4B4 (10 μg/ml) where indicated and plated on α-uPAR antibody R4 (20 μg/ml) for 30 minutes. Cell area was quantified from DIC images and data are represented as mean 95% c.i., n=50 in two independent experiments Upper and lower grey area represents respectively the range of fully or not spread cells, based on the confidence intervals retrieved in Fig. 2.

The apparent lack of structural requirements to the receptor and its matrix ligand suggests that the sole requirement to these structures is that they physically connect the cell to the matrix. In favor of this mode of action, both VN^{RAD} and the anti-uPAR antibody failed to induce p130Cas activation when presented to non-adherent cells in a soluble form (Fig. 17 "off-plate").

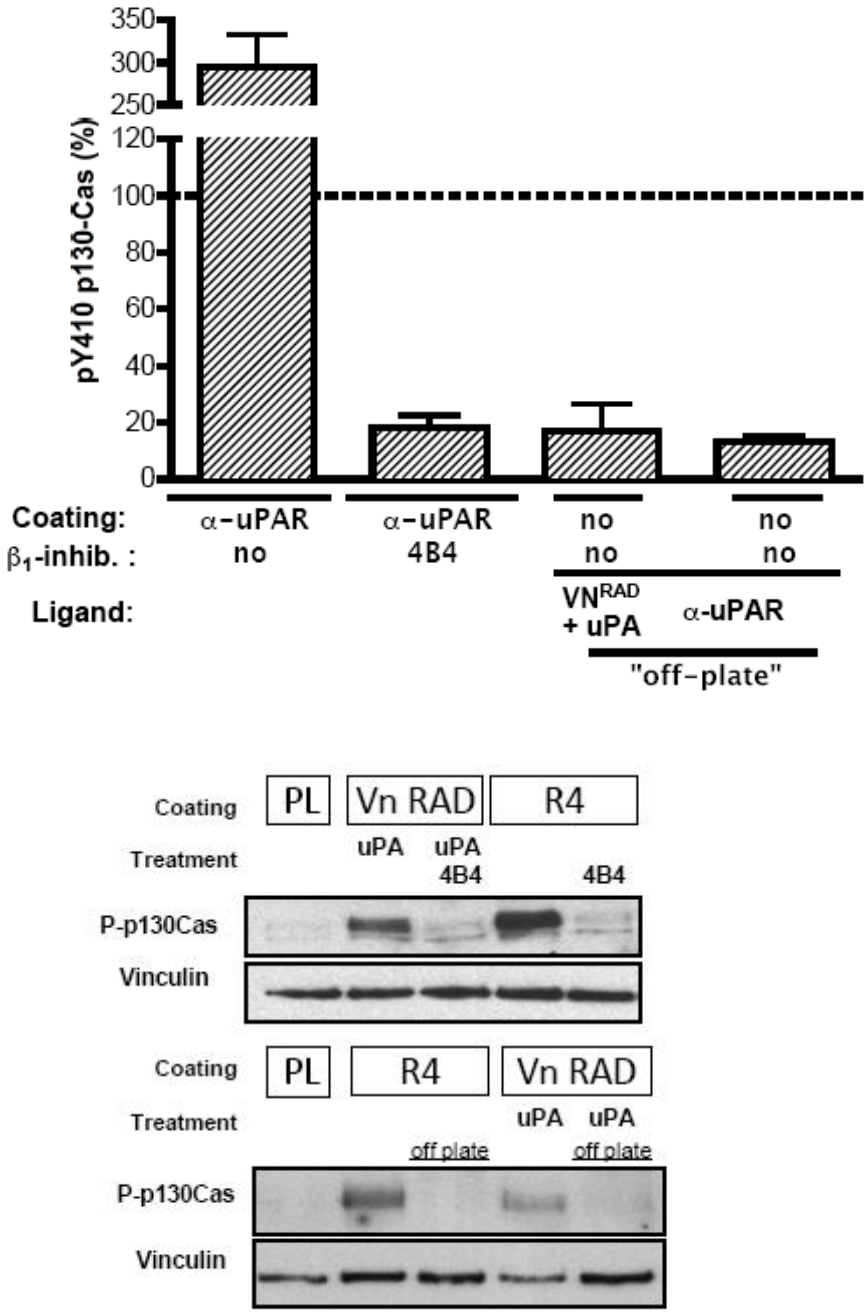


Figure 17 uPAR-R4 interaction triggers β_1 integrin dependent p130Cas phosphorylation. Western blot and densitometric analysis: $uPAR^{T54A}$ cells were pre-incubated with antibody 4B4 (10 μg/ml) where indicated and plated on α-uPAR antibody R4 (20 μg/ml), PL (100 μg/ml) or VN^{RAD} (10 μg/ml) for 30 minutes. p130Cas phosphorylation was quantified from WB and expressed as percentage of phosphorylation of 293 $uPAR^{T54A}$ on VN^{RAD} with uPA, data are means ± s.e.m., n=3. Off-plate condition refers to experiment where the coating components were added to non adhering cells. Representative western blot are shown.

This indicates that the mere binding of soluble proteins to uPAR is not proficient in triggering changes in signal transduction. On the contrary, these molecules have to be attached to a substrate providing rigidity and cell adhesion in order to induce ligand-independent integrin signaling. To prove this we performed experiments incubating non-adherent cell with Dynabeads coated with different substrates. Dynabeads coated with FN produced a strong p130Cas phosphorylation in uPAR^{T54A} cells, compared with beads coated with PL. This is consistent with a canonical integrin outside-in signaling, proving that the ligands attached to Dynabeads are functional. Interestingly uPAR-VN (uPAR^{T54A} cells treated with uPA) interaction triggers p130Cas phosphorylation also when VN^{RAD} presented by Dynabeads. uPAR-VN induced p130Cas phosphorylation was abrogated by 4B4 treatment (Fig. 18), demonstrating that, like in the case of VN^{RAD} coated on cell culture plates, the signaling derives from β_1 integrin and does not require integrin-matrix interaction. In these experiments the ratio between cells and beads is 1:2, meaning that every cell will bind two beads on average. This data could indicate that "thinly localized" uPAR-mediated VN adhesion can trigger integrin signaling, independently of integrin-matrix interaction.

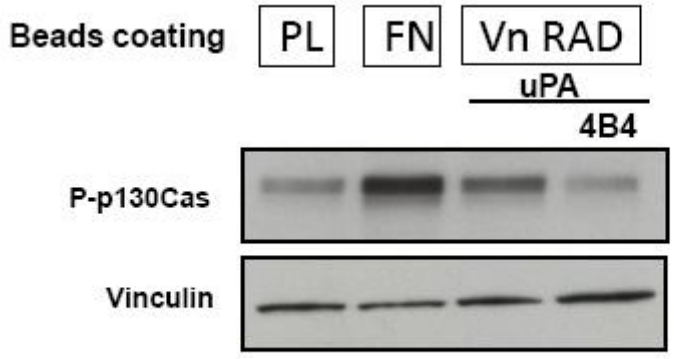


Figure 18 VN^{RAD} presented by Dynabeads induces integrin signaling to p130Cas. Western blot analysis: uPAR^{T54A} cells were incubated with dynabeads coated with PL, FN or VN^{RAD} for 30 minutes in agitation at 37°C. uPA (10 nM) and 4B4 (10 μg/ml) were added where indicated. Cells attached to the beads were lysed and p130Cas was assayed. Representative blots are shown.

Both uPAR and the artificial PAI-1_{GPI} receptor are tethered to the cell membrane by a GPI-anchor suggesting that the particular properties of this anchor, such as the preferential partitioning to membrane sub-domains known as lipid rafts, could contribute to the observed biological activity. Furthermore, different GPI-anchoring sequences have been shown to endow their attached ectodomains with different biological activities (Paulick and Bertozzi, 2008). To determine the importance of the type of membrane anchorage on the biological effects under study, we generated five variants of $uPAR^{T54A}$ with different C-terminal membrane anchorage sequences. Two of these were engineered to use the GPI-anchoring signal from the GPI-anchored isoform of neuronal cell adhesion molecule (NCAM, $uPAR^{NCAMgpi}$) and from the carcinoma embryonic antigen S4 (CEA-4S, $uPAR^{CEAgpi}$). These GPI-anchoring sequences were chosen because they have been published to "encode" different biological properties to the attached GPI-anchor. In addition we generated three variants of uPAR tethered to the membrane by transmembrane domains copied from the epidermal growth factor receptor (EGFR, $uPAR^{EGFRtm}$), NCAM ($uPAR^{NCAMtm}$) or CEA ($uPAR^{CEAtm}$). These transmembrane domains were trimmed to retain only a few cytoplasmic residues to limit the possible binding of cytoplasmic proteins. The different receptor chimeras were transfected into 293 Flp-In T-Rex cells and assayed for their ability to induce cell adhesion, spreading and p130Cas SD phosphorylation after seeding on VN^{RAD}. Briefly, we found that all the different chimeras were comparably expressed on the cell surface (data not shown) and induced similar levels of cell adhesion to VN^{RAD} (Fig.19).

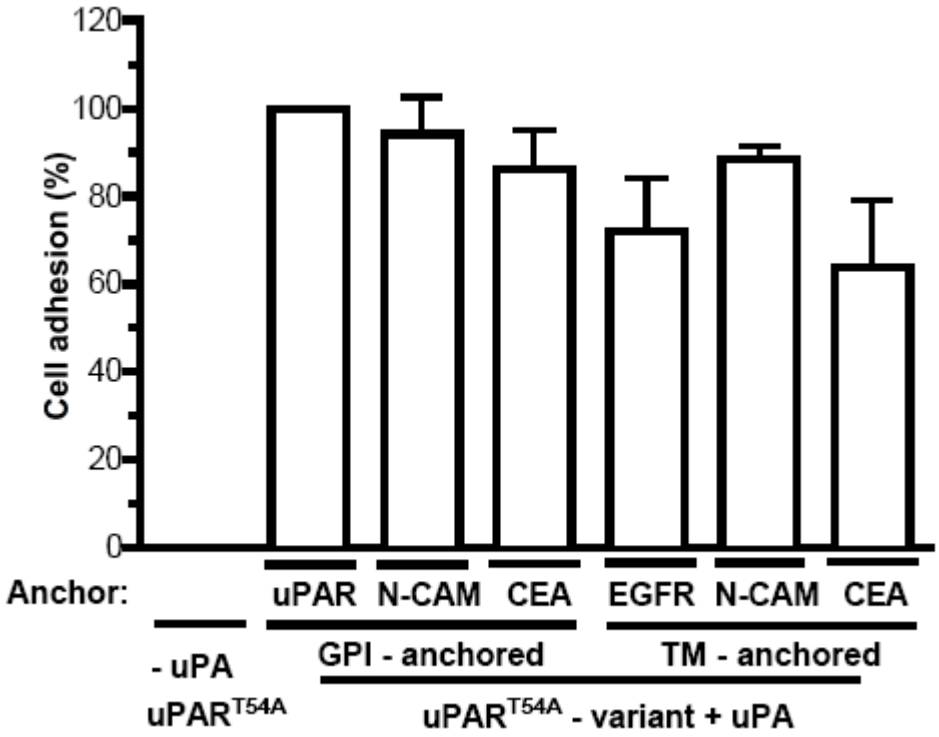


Figure 19 The mean of anchorage to the plasma membrane does not affect uPAR-mediated VN adhesion. Cell-adhesion assay: cell expressing the indicated uPAR variant were plated on VN^{RAD} (5 μg/ml) with or without uPA (10 nM) for 30 minutes. Cell were fixed and stained with crystal violet. Cell adhesion was quantified and expressed as percentage of Poly-D-lysine adhesion. Data are expressed as mean ± s.e.m, n=3.

Moreover, the different means of membrane anchorage did not influence the ability of the receptors to induce p130Cas phosphorylation (Fig. 20) and cell spreading (Fig. 21) on VN^{RAD}. These data demonstrate that both the type and the exact sequence of the membrane anchorage signal are of no or little importance for uPAR to induce β_1-dependent signaling to p130Cas and downstream cell spreading.

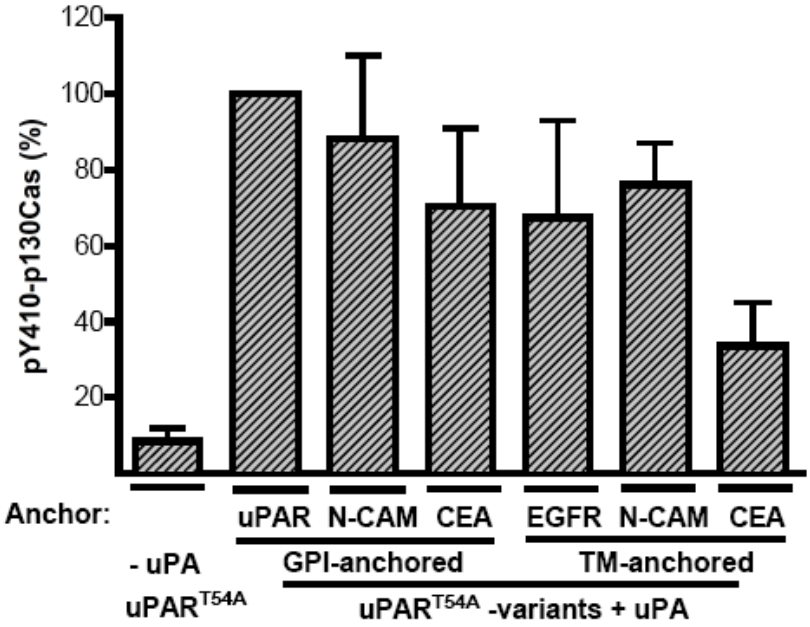


Figure 20 uPAR-mediated p130Cas phosphorylation is not affected by the mean of anchorage to the plasma membrane. Western blot and densitometric analysis: cell expressing the indicated uPAR variant were plated on VN^{RAD} (5 μg/ml) with uPA (10 nM) for 30 minutes. p130Cas phosphorylation was quantified from WB and expressed as percentage of phosphorylation of 293 $uPAR^{T54A}$ on VN^{RAD} with uPA, data are means ± s.e.m., n=3.

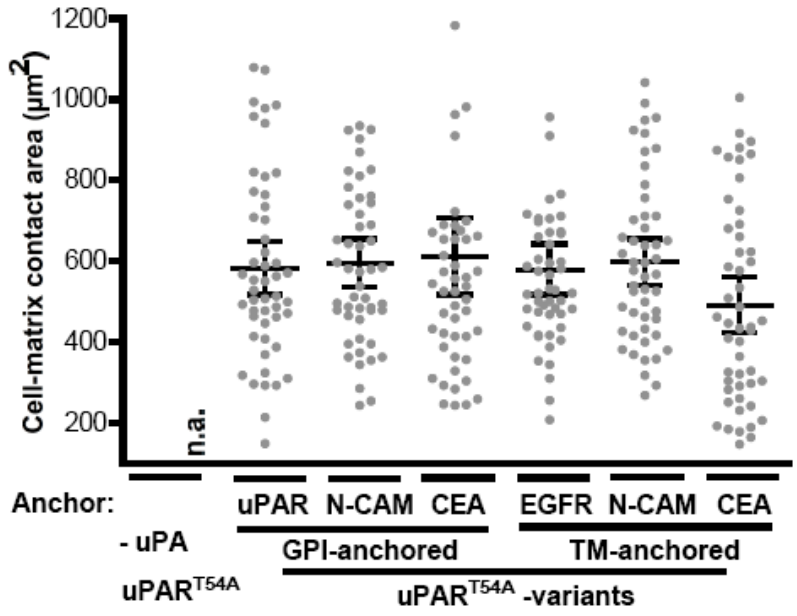


Figure 21 All the different anchored uPAR variants induce cell spreading. Quantification of cell-matrix contact area: cell expressing the indicated uPAR variant were plated on VN^{RAD} (5 μg/ml) with uPA (10 nM) for 30 minutes. Cell area was quantified from DIC images and data are represented as mean 95% c.i., n=50 in two independent experiments Upper and lower grey area represents respectively the range of fully or not spread cells, based on the confidence intervals retrieved in Fig. 2.

uPAR-induced β_1 outside-in signaling requires the binding of cytoplasmic factors, an active conformation, but occurs independently of ligand-engagement

The uPAR/VN-induced signaling via $\alpha_V\beta_5$ on VN is coherent with the current paradigm for ligand-induced integrin outside-in signaling and we therefore focused our attention to the ligand-independent β_1-mediated signaling observed in cells seeded on VN^{RAD}. Both mAb13 and 4B4 belong to a family of allosteric inhibitory β_1 antibodies that are thought to stabilize the receptor in an inactive conformation by preventing the swing-out of the hybrid domain (Luo et al., 2004b). The fact that these antibodies inhibit adhesion-induced cell spreading and signaling on a non-integrin substrate suggests that signaling requires an active conformation of β_1 integrin, irrespectively of the ligand binding properties of this conformation. To address this possibility directly we established a simple cell system to conduct structure-function analysis on β_1 in uPAR/VN-signaling. For this, we first generated a stable clone of 293 Flp-In T-REx cells expressing the $uPAR^{T54A}$ receptor by transfection and G418 selection. This clone (termed 293^{uT54Ac}) was selected to express a level of receptor comparable to that obtained after tetracycline induction of the Flp-In T-REx cell lines used in the preceding experiments and was found to result in qualitatively and quantitatively similar effect on cell signaling and changes in cell morphology upon treatment with uPA. This cell line was subsequently used for assaying the biological activity of modified β_1-chains introduced by Flp-In transfection. To discriminate the biological activity of the transfected β_1-chains from the activity of residual endogenous β_1 we rendered the transfected β_1-chain refractory to inhibition by the 4B4 antibody (but still

sensitive to the effect of mAb13) using the K218A substitution that has been reported to impair antibody binding (Luo et al., 2004b). To functionally validate this system we conducted FN-adhesion assays on cells that were transfected with either empty vector or with vectors encoding β_1^{WT} and β_1^{K218A} in the presence or absence of the inhibitory mAb13 and 4B4 inhibitory antibodies (Fig. 22). As predicted, both antibodies strongly reduced FN-adhesion of mock and β_1^{WT} transfected cells while only mAb13 inhibited FN-adhesion of cells expressing β_1^{218A}, demonstrating that this β_1-chain is resistant to inhibition by 4B4, but otherwise functionally normal.

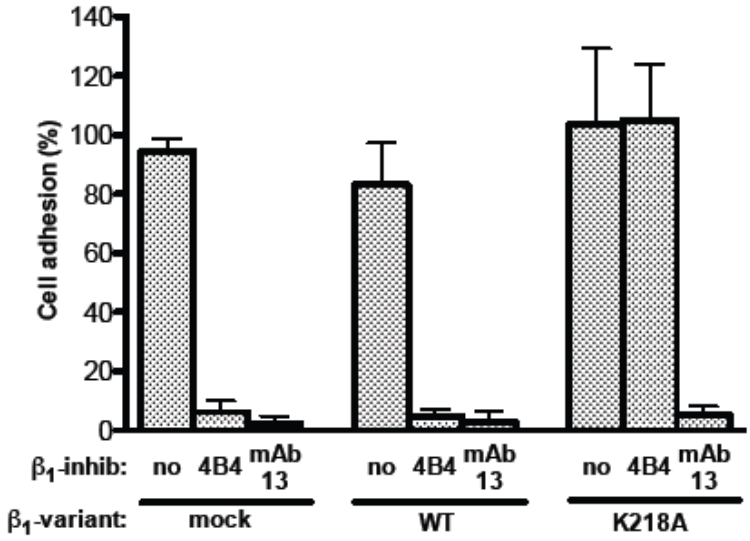


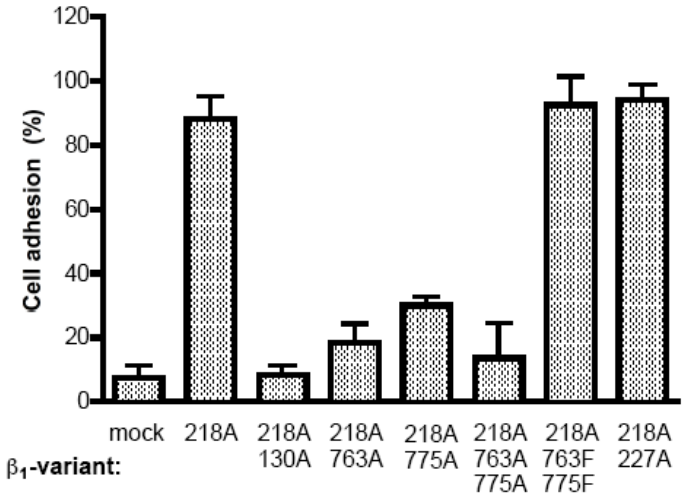
Figure 22 K218A mutation makes β_1 integrin insensitive to 4B4 antibody inhibition. Cell-adhesion assay: 293 $uPAR^{T54A}$ cells expressing WT β_1 integrin or k218A mutant were plated on FN (10 μg/ml) in presence on two β_1 integrin allosteric inhibitory antibodies (4B4 and mab13, 5 μg/ml) for 30 minutes. Cell adhesion was quantified and expressed as percentage of poly-D-lysine one.

As consequence, by performing the adhesion, spreading and signaling assays in presence of 4B4 antibody, the only "functional" integrin receptors expressed on the cell surface will be the ones containing the mutated β_1 subunit. The K218A mutation will be

combined with other mutations within the extracellular part or in the cytoplasmic tail, known to affect β_1 integrin function.

Using this system, we first sought complementary evidence for the ligand independence of the β_1-signaling induced by the uPAR/VN^{RAD}-interaction. To this purpose we utilized the alanine substitution D130A (Takada et al., 1992) that disrupts the metal ion dependent adhesion binding site (MIDAS) in β_1 resulting in greatly abolished binding of β_1 matrix-ligands. Indeed, expression of $\beta_1{}^{218/130A}$ strongly abrogated cell adhesion to FN (Fig. 23a) as well as downstream p130Cas SD phosphorylation (Fig. 24a), meaning that this integrin mutant is not able to contact the extracellular matrix. The effect of the D130A mutation was not restricted to FN as it also prevented cell adhesion to other β1 substrates including laminin and collagen (data not shown). The effect of the D130A mutation was however specific and restricted to β_1-integrins as cell adhesion to VN, mediated by $\alpha_V\beta_5$, remained unaffected (data not shown). $\beta_1{}^{130/218A}$ mutant did not alter cell adhesion to VN^{RAD} in presence of uPA, being mediated exclusively by uPAR (Fig. 23b).

Fig. 23a (FN adh.)



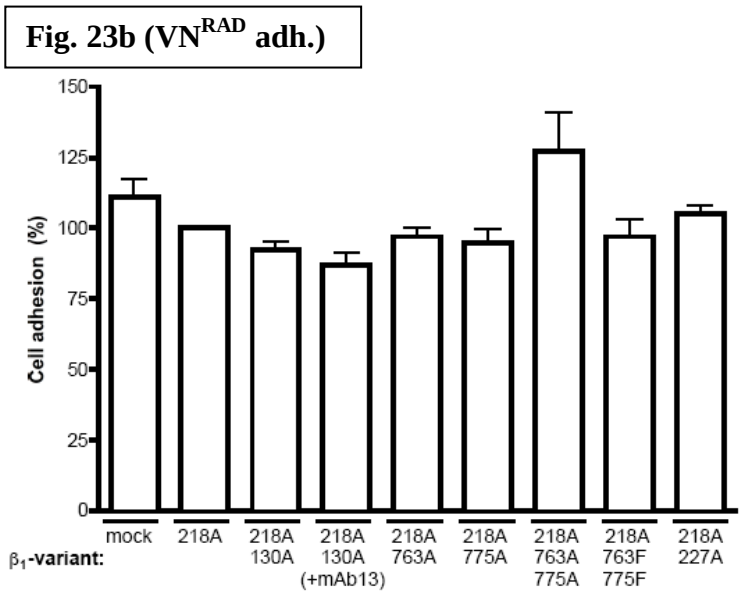


Figure 23 Effect of β_1 integrin mutations on integrin-dependent and uPAR-mediated cell adhesion. Cell-adhesion assay: cell expressing the indicated β_1 integrin variants were pre-incubated with 4B4 antibody (10 μg/ml) and plated for 30 minutes on either FN (10 μg/ml, **Fig. 23a**) or VN^{RAD} (5μg/ml, **Fig. 23b**) with uPA. Cell adhesion was quantified and expressed as percentage of poly-D-lysine one. Both sets of data are represented as mean ± s.e.m., n=3.

Importantly the $\beta_1{}^{130/218A}$ chain was still capable of transmitting the uPAR/VN^{RAD}-induced signaling to p130Cas SD phosphorylation (Fig.24b) and enhancing cell spreading (Fig. 25), documenting conclusively that β_1 matrix engagement is dispensable for transduction of uPAR-signaling. Importantly, the uPAR/VN^{RAD}-signaling transduced by the D130A mutant was fully suppressed by the allosteric inhibitory β_1-antibody mAb13 (Fig. 24b and 25 mAb13) demonstrating that the activity of this antibody, and presumably also that of 4B4, is not related to its effect on ligand binding *per se*, but rather to some other conformation-dependent biological activity of the integrin.

Fig. 24a (FN sig.)

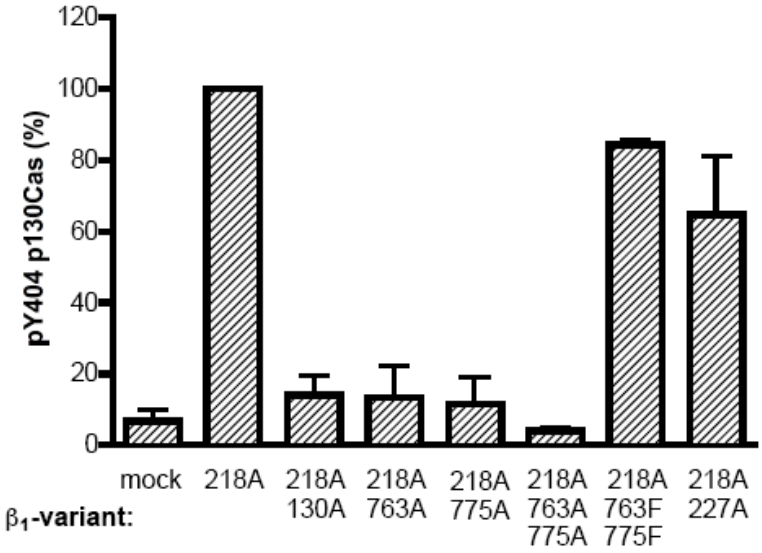


Fig. 24b (VN^{RAD} sig.)

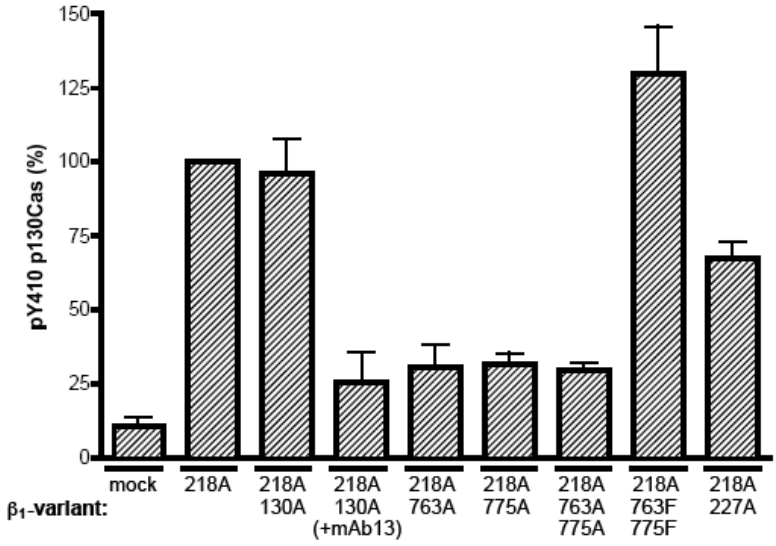


Figure 24 Structural requirements for ligand-independent β_1 integrin signaling and canonical integrin-mediated outside-in signalling to p130Cas**.** Western blot and densitometric analysis: 293 $uPAR^{T54A}$ cells expressing the indicated integrin variant were pre-treated with 4B4 antibody (10 μg/ml) and Mab13 antibody where indicated (10 μg/ml, where indicated) and seeded on VN^{RAD} (10 μg/ml, **Fig. 24b**) in presence of uPA (10 nM) or FN (10 μg/ml, **Fig 24a**). P130Cas phosphorylation in Y410 was assayed and expressed setting the level of K218A expressing cells as 100%. Both sets of data are represented as mean ± s.e.m., n=3. Representative western blot are shown.

The conformation and activation state of integrins is largely determined from the intracellular side of the plasma membrane through the binding of scaffolding proteins to the cytoplasmic tails of integrins (Legate and Fassler, 2009) and we therefore also analyzed the structural requirements to this region of β_1 integrin in uPAR/VN^{RAD}-signaling. We focused our attention on the two NPxY motifs that are widely accepted to be critical for integrin function because of their interactions with members of the talin and kindlin families of scaffolding proteins (Moser et al., 2009a) (Montanez et al., 2008) (Czuchra et al., 2006) (Tadokoro et al., 2003). For this purpose we generated β_1-mutants in which we had alanine-substituted the tyrosines of the membrane-proximal ($\beta_1^{218A/Y763A}$) and the membrane-distal ($\beta_1^{218A/Y775A}$) NPxY-motifs alone or in combination ($\beta_1^{218A/Y763A\ Y775A}$), as well as a control mutant in which both tyrosines were replaced with the permissive double phenylalanine substitutions ($\beta_1^{218A/763/775F}$). Control experiments assaying the adhesion and signaling activity of the mutated β_1-chains in cells seeded on FN demonstrated that alanine substitution in either of the two NPxY-motifs efficiently abrogates cell adhesion (Fig. 23a) and subsequent p130Cas SD phosphorylation (Fig. 24a), while the double phenylalanine substitution retained full functionality. We next assayed uPAR/VN^{RAD}-induced cell adhesion (Fig. 23b), spreading (Fig. 25) and p130Cas SD phosphorylation (Fig. 24b) on VN^{RAD}. In analogy to the experiments on FN, we found that alanine substitutions in the NPxY-motifs very efficiently inhibited both cell spreading and p130Cas SD phosphorylation, while double phenylalanine substitution did not alter signal transmission and cell morphology changes. Cell adhesion to VN^{RAD}, however, was not affected by these β_1-mutations and the lack of spreading and signaling therefore, unlike on FN, cannot be attributed to impaired cell adhesion.

In order to consistently exclude a possible direct lateral interaction between uPAR and β_1 integrin we performed experiments using a mutant ($\beta_1^{218A/S227A}$) reported to destroy

this interaction (Wei et al., 2005). This integrin mutant had no defects in supporting FN adhesion (Fig. 23a) and the consequent p130Cas phosphorylation (Fig 24a). When tested in cells adhering to VN^{RAD} through uPAR in presence of 4B4, $\beta_1{}^{218A/S227A}$ induced p130Cas phosphorylation (Fig. 24b) and enhanced cell spreading (Fig 25). These data finally rule out a possible direct uPAR-integrin interaction in the signaling we are observing.

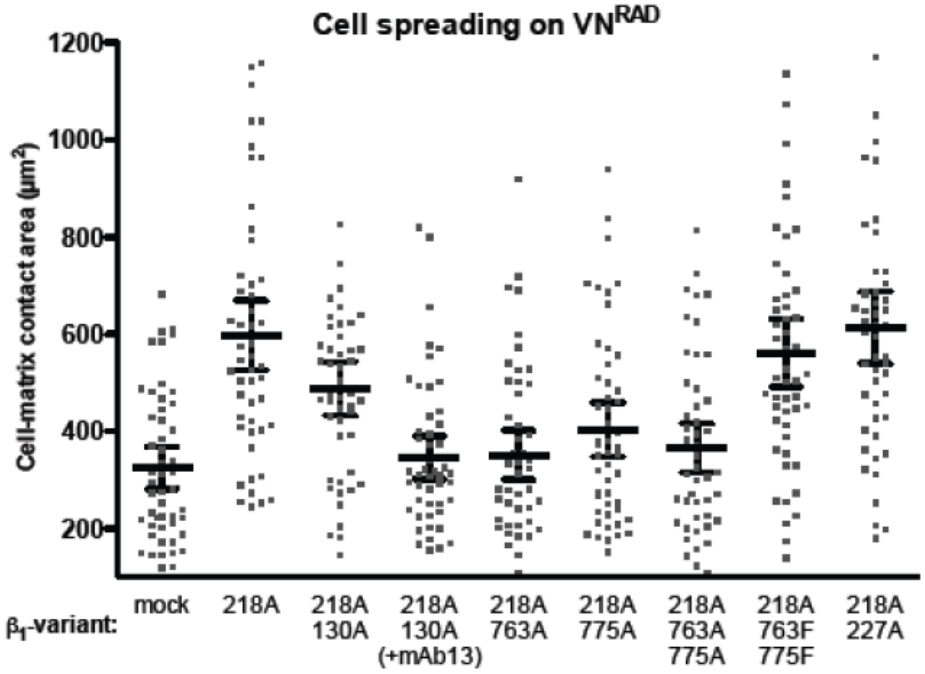


Figure 25 Effect of β_1 integrin mutations on ligand-independent β_1 integrin signalling. Quantification of cell-matrix contact area: 293 $uPAR^{T54A}$ cells expressing the indicated integrin variant were pre-treated with 4B4 antibody (10 µg/ml) and Mab13 antibody where indicated (10 µg/ml, where indicated) and seeded on VN^{RAD} (5 µg/ml) in presence of uPA (10 nM). Cell area was quantified from DIC images and data are represented as mean 95% c.i., n=50 in two independent experiments. Upper and lower grey area represents respectively the range of fully or not spread cells, based on the confidence intervals retrieved in Fig. 2.

In conclusion, these data show that the activity of β1 in transducing adhesion-induced cell spreading and signaling is independent of ligand-binding, but requires an

active conformation of the integrin as well as the binding of cytoplasmic adaptor proteins like talin and kindlin.

Ligand-independent outside-in signaling downstream of β3

uPAR-mediated VN adhesion triggers ligand-independent β_1 integrin signaling. However, excluding a direct lateral interaction between these membrane receptors ((Madsen et al., 2007) and see below), this mechanism could be more general and not only restricted to one class of β integrin subunits.

To validate this hypothesis 293^{uT54Ac} were transfected with constructs carrying β_3 integrin variants. Adhesive properties of the cells obtained in this way were assayed (Fig. 26) revealing that β_3^{WT} integrin expression does not affect FN adhesion and basal or uPAR-mediated cell adhesion to VN^{RAD}. On the contrary, VN adhesion (integrin dependent without uPA) was highly increased, suggesting that β_3 integrin subunit couples with endogenous α_V to form α_V β_3 vitronectin receptor. The expression of β_3^{119Y} and $\beta_3^{747/759A}$ integrin mutants, that respectively compromise ligand binding activity (Loftus et al., 1990) and the two NPxY-motifs crucial in the interaction with talin and kindlin (Moser et al., 2009b) required for integrin activation, strongly decreased VN adhesion resulting in levels lower than mock transfected cells. This is well explained by the competitive effect that over-expressed β_3 integrin expression exerts on the endogenous β_5 subunit.

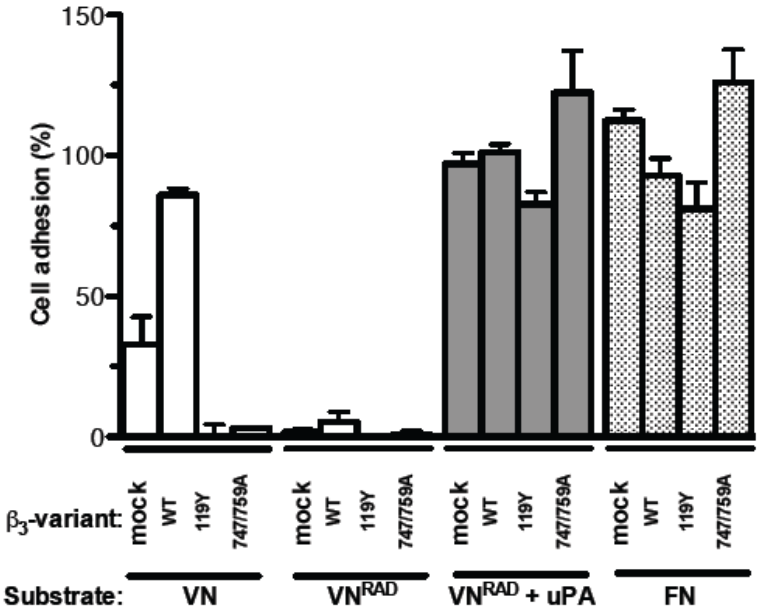


Figure 26 Adhesive properties of 293 uPAR^{T54A} cells expressing β_3 integrin mutants. Cell-adhesion assay: Cells were seeded on FN (10 μg/ml), VN and VN^{RAD} (5 μg/ml) with uPA (10 nM) where indicated. Quantified cell adhesion was expressed in percentage of poly-D-lysine one. Data are means ± s.e.m., n=3.

We then analyzed if β_3 integrin could transmit uPAR-VN signaling with similar structural requirements to β_1 integrin. To this purpose, we measured p130Cas phosphorylation and cell spreading in cells expressing the integrin mutants mentioned above, seeded on VN^{RAD} with uPA. β_1 integrin was inhibited with 4B4 antibody, in order to study specifically the signaling deriving from β_3 integrin. uPAR-mediated signaling and cell spreading on VN^{RAD} was efficiently supported by both $\beta_3{}^{WT}$ and $\beta_3{}^{119Y}$ (even when β_1 integrin signaling was blocked by 4B4 treatment). The Mutation in both the NPxY-motifs in integrin cytoplasmic tail abolished uPAR-VN signaling resulting in low levels of p130Cas SD phosphorylation (Fig. 27) and cell spreading (Fig. 28). This structure-function analysis indicates that ligand binding is dispensable in uPAR/VN-induced signaling and cell spreading through β_3 integrin, while cytoplasmic interactions occurring in the two NPxY-motifs with kindlin and talin are crucial in this process. Moreover, this ligand-independent adhesion-induced signaling does not seem to be specifically related to

β_1 integrin subunits, as it is efficiently recapitulated by β_3-containing integrins. Our data points toward a more general mechanism with precise structural-function requirements.

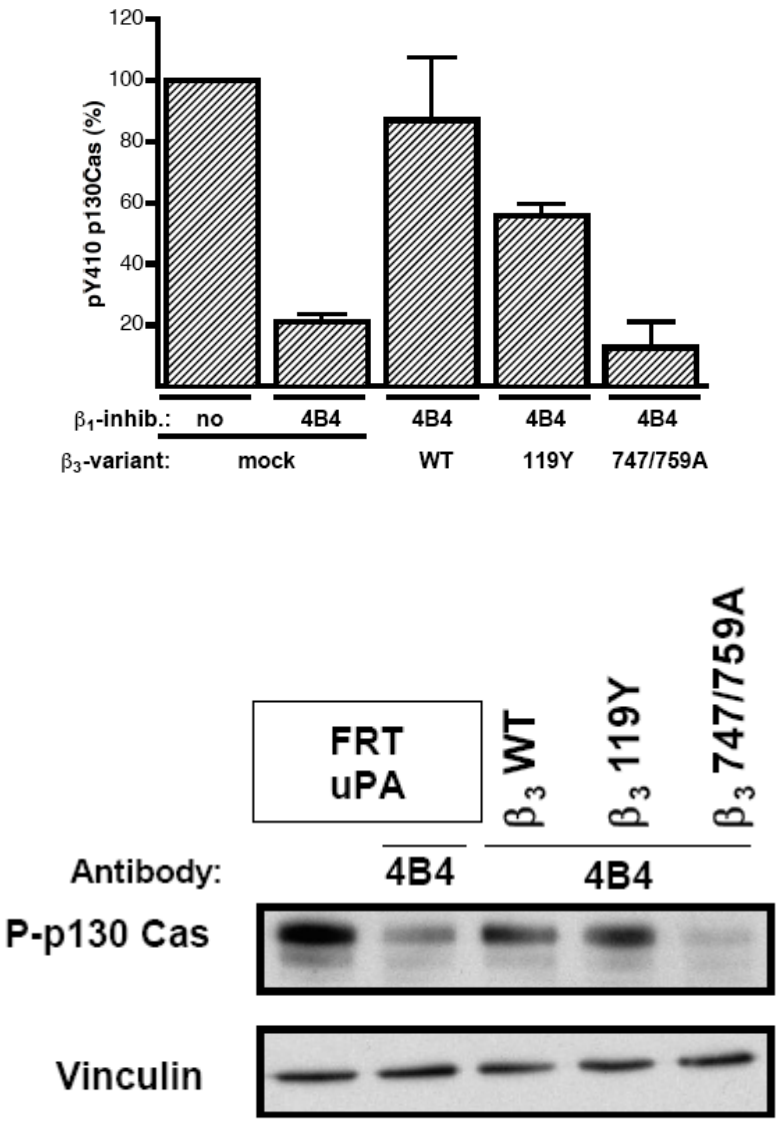


Figure 27 β_3 integrin transmits ligand-independent integrin signaling downstream of uPAR-VN interaction. Western blot and densitometric analysis: upon pre-incubation with 4B4 antibody (10 μg/ml) cells were plated for 30 minutes on VN^{RAD} (10 μg/ml) in presence of uPA (10 nM). p130Cas phosphorylation was assayed and quantified. Western blot data were normalized setting phosphorylation level of mock transfected cells without inhibitory antibody as 100% and expressing them as means ± s.e.m. , n=3. Representative western blot are shown.

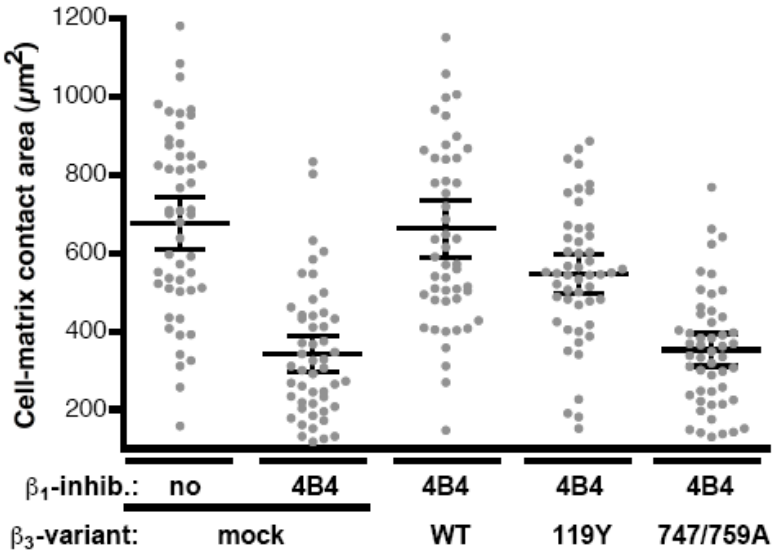


Figure 28 Ligand binding is dispensable for β_3 integrin induced cell spreading downstream of uPAR-VN interaction. Quantification of cell-matrix contact area: Upon pre-incubation with 4B4 antibody (10 μg/ml) cells were plated for 30 minutes on VN^{RAD} (10 μg/ml) in presence of uPA (10 nM). Cell-matrix contact area of DIC images was quantified. DIC data are represented as mean ± 95% c.i., n=50 in two independent experiments.

Ligand-independent trans-activation may occur between different β-integrin subunits

Our data are consistent with a model where the anchoring-adhesive receptor differs from the signaling one. If this model is correct, it should be valid even when the anchoring and the signaling receptor are two different kinds of integrins. Thus a signaling incompetent integrin, capable of mediating cell adhesion, should signal through another integrin unable to engage the extracellular matrix. To achieve this we plated β_3 integrin expressing cells on anti-β_3 antibody. As consequence even an integrin mutant that cannot interact with the

two main integrin activators (talin and kindlin, $\beta_3^{747/759A}$) should still support cell adhesion without directly transmitting a signal.

β_3 integrin expressing cells strongly adhered to anti-$\alpha_v\beta_3$ antibody (LM09) coated plates (Fig. 29), while mock transfected cells did not, meaning that the endogenous integrins array expressed by 293 cells, and specially β_1-containing integrins, are unable to interact with this artificial coating. Importantly $\beta_3^{747/759A}$ integrin mutant, despite the missing interaction with kindlin and talin, was still capable of mediating cell adhesion to anti-$\alpha_v\beta_3$ antibody coated plates.

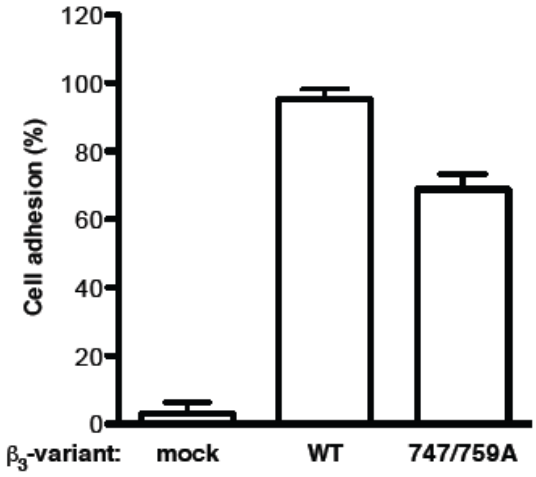


Figure 29 β_3 integrin expressing cells adhere to coated anti-β_3 integrin antibody. Cell-adhesion assay: Cells expressing the indicated integrin mutant were seeded on anti-$\alpha_v\beta_3$ (LM09) coated plates (20 μg/ml). Cell adhesion was expressed as percentage of poly-D-lysine. Data are means ± s.e.m., n=3

The mechanical interaction between β_3^{WT}integrin and LM609 induced cell spreading (Fig. 31) and p130Cas phosphorylation (Fig. 30). This signaling was not blunted by β_1 integrin inhibition consistently with a canonical outside-in signaling from β_3 integrin. Importantly, even if deprived of its key interactors, the $\beta_3^{747/759A}$ integrin was still capable of inducing cell spreading and signaling upon LM609 interaction.

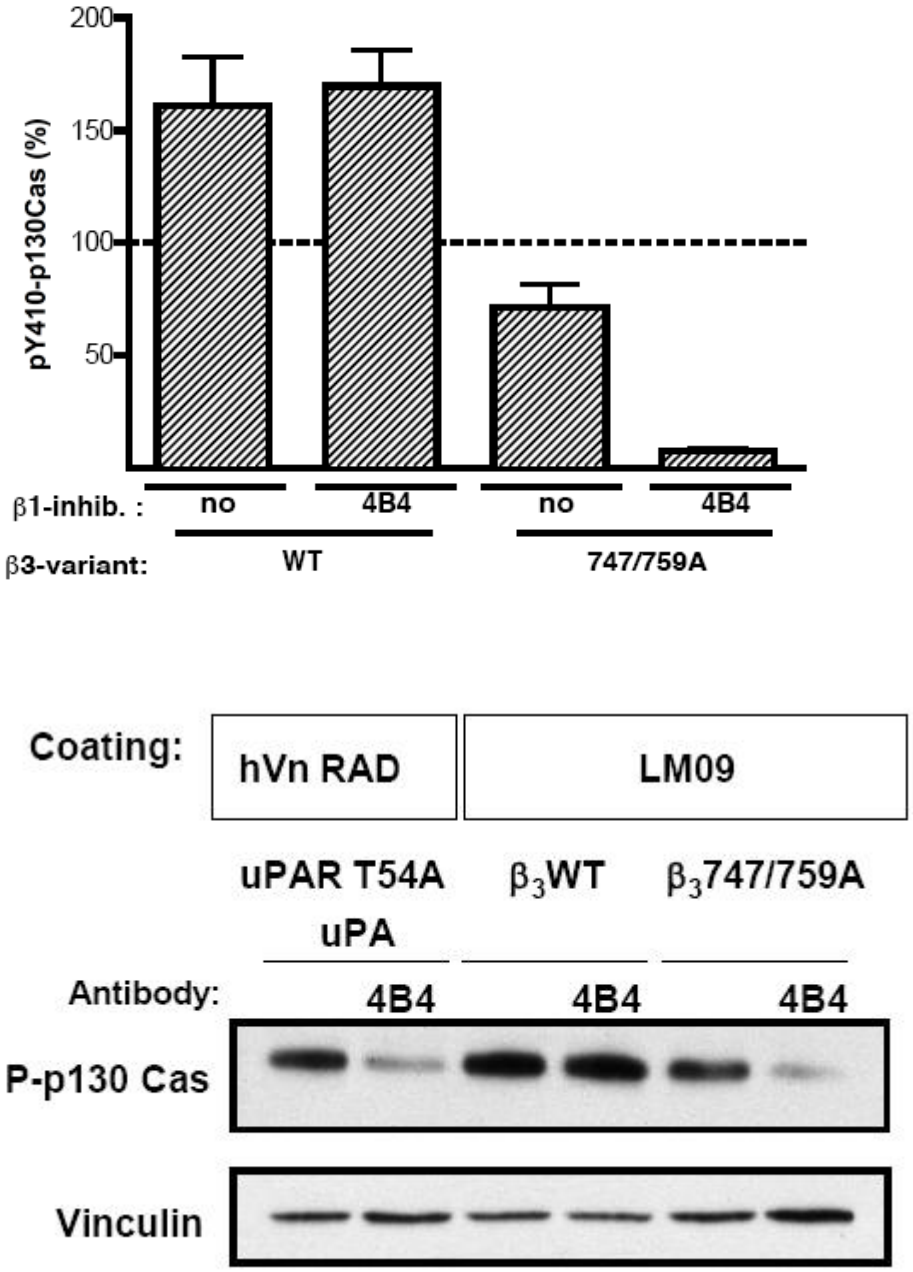


Figure 30 A signaling-incompetent β_3 integrin mutant induces cell adhesion to LM09 triggering β_1 integrin dependent signalling to p130Cas. Western blot and densitometric analysis. Cell expressing the indicated β_3 integrin (pre-incubated with 4B4 (10 μg/ml) where indicated) were plated for 30 min on LM09 (20 μg/ml) coated plates prior to lysis. P130cas phosphorylation was assayed and quantified. Data are normalized setting the level in 293 $uPAR^{T54A}$ cells on VN^{RAD} with uPA as 100%. Data are means ± s.e.m., n=3. Representative western blot are shown.

Importantly, $\beta_3^{747/759A}$ integrin induced signaling and cell spreading was inhibited by β_1 integrin blockage achieved through 4B4 treatment (Fig. 30 and Fig. 31). These data demonstrate that a signaling incompetent integrin can still mediate mechanical adhesion to anti-$\alpha_v\beta_3$ antibody. The adhesive signaling deriving from this interaction is transmitted by β_1 integrin that, without engaging the ECM, induce changes in cell morphology and intracellular signaling. Thus integrins can carry out both anchoring and signaling receptor function, suggesting that ligand-independent adhesive signaling may occur also between different integrin types.

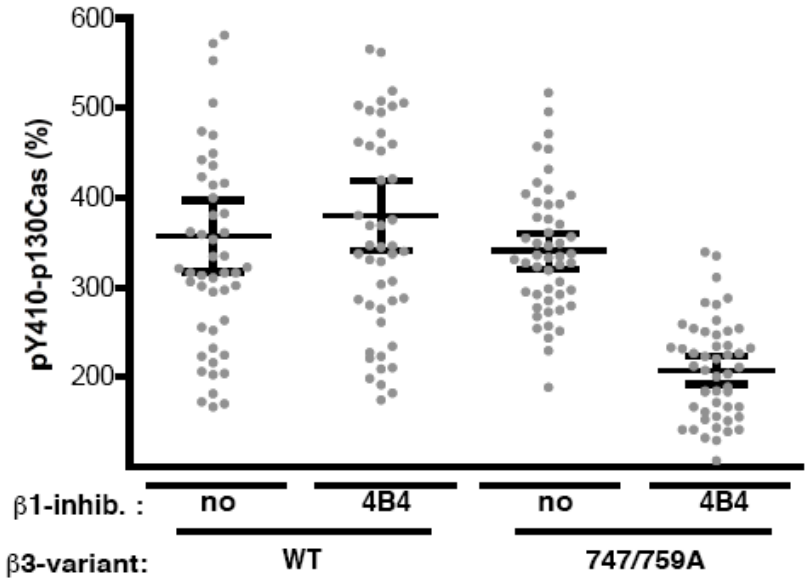


Figure 31 β_3 mechanical cell adhesion induces cell spreading through β_1 integrin. Quantification of cell-matrix contact area. Cell expressing the indicated β_3 integrin (pre-incubated with 4B4 (10 μg/ml) where indicated) were plated for 30 min on LM09 (20 μg/ml) coated plates prior to fixation. DIC images were quantified. Cell-matrix contact area data are means ± 95% c.i., n=50 in two independent experiments.

Ligand-independent integrin signaling requires SRC activity to induce cell spreading and migration:

Cell migration is dynamic, strongly regulated process that involves a tight compartmentalization of intracellular signaling and a continuous FAs turnover (Ridley et al., 2003), where integrins mediate cell adhesion and transmit signaling(s) across the plasma membrane. uPAR-VN interaction has been shown to strongly increase cell migration (Madsen et al., 2007; Smith et al., 2008) (Kjoller and Hall, 2001). However, the importance of integrin-ECM engagement in this process was not investigated.

The importance of integrin-matrix interaction in uPAR-induced cell migration was therefore evaluated on a VN variant with destroyed integrin binding site. $uPAR^{T54A}$ cells were seeded on VN^{RAD} in presence of uPA and cell migration was quantified from timelapse movies. Even on a substrate with mutated integrin binding site, cells managed to migrate showing that not only cell adhesion and spreading but also cell migration was not impaired by the RGD motif mutation (Fig. 32).

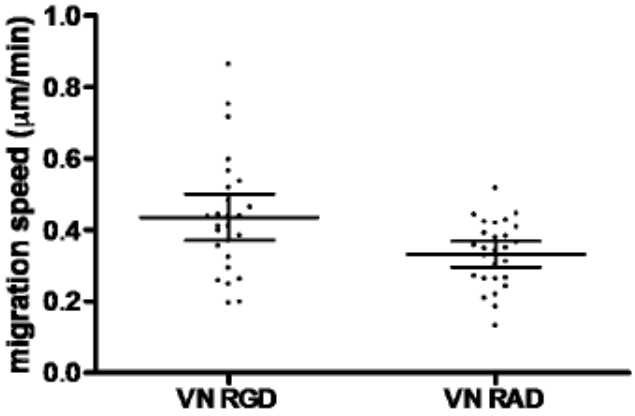


Figure 32 uPAR-VN induced cell migration is independent of integrin-matrix interaction. Quantification of migration speed. 293 $uPAR^{T54A}$ cells were seeded on VN or VN^{RAD} (10 μg/ml) in presence of uPA (10nM). After 1 hours time lapse recordings were started and cell migration speed was quantified. Data are expressed as means ± 95% c.i., n=25 in two independent experiments

Moreover, uPAR-VN interaction transduces a ligand-independent β_1 integrin signaling and this signaling could be responsible for uPAR-induced cell migration as well. To test this possibility cell migration was quantified upon β_1 integrin inhibition through 4B4 treatment. As expected 4B4 treatment, by having an evident inhibitory effect on cell spreading, strongly impaired cell migration on VN^{RAD} (Fig. 33).

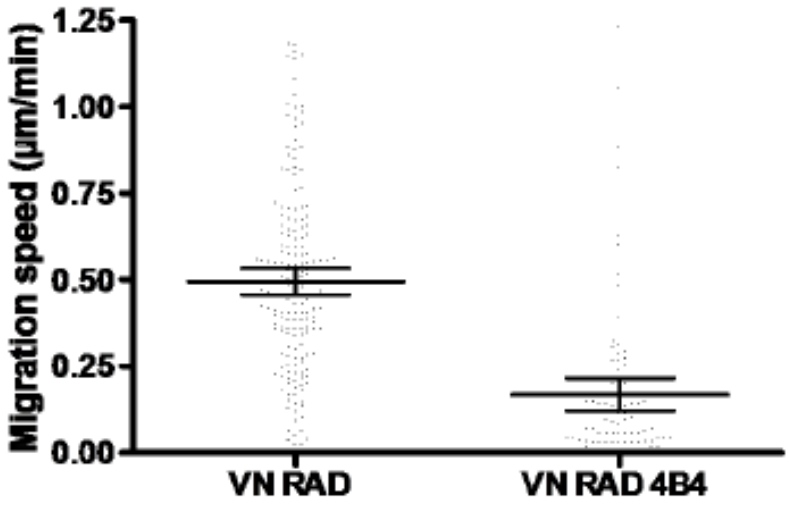


Figure 33 uPAR-VN induced cell migration does not require integrin-matrix interaction and it is mediated by β_1 integrin. Quantification of migration speed. 293 $uPAR^{T54A}$ cells, pre-treated with 4B4 (10 µg/ml) for 15 minutes where indicated, were seeded on VN^{RAD} (10 µg/ml) in presence of uPA (10nM). After 1 hours time lapse recordings were started and cell migration speed was quantified. Data are expressed as means ± 95% c.i., n=50 in two independent experiments

These data demonstrate that integrin-matrix interaction is dispensable in cell migration triggered by uPAR-mediated VN adhesion. Moreover, the same ligand-independent signaling, responsible for enhanced cell spreading, seems to be sufficient to induce cell migration also on substrates refractory to integrin engagement.

Since the predominant signaling pathway induced by uPAR-mediated VN adhesion is ligand-independent β_1 integrin signaling we decided to better study this new mechanism of signal transduction. To do that we targeted the proteins involved in uPAR signaling (like p130cas and ERK1/2) and possible kinases up-stream of them through chemical inhibitors. $uPAR^{T54A}$ cells, pre-incubated with specific inhibitors, were plated on VN^{RAD} in presence of uPA. Two Src family kinase inhibitors (PP1 and PP2) efficiently impaired uPAR-induced p130Cas and ERK1/2 phosphorylation, while the inactive form of the inhibitor (PP3) had no effect. The specific MEK inhibitor (UO1026) completely abolished ERK1/2 phosphorylation but had no effects on p130Cas phosphorylation (Fig 34).

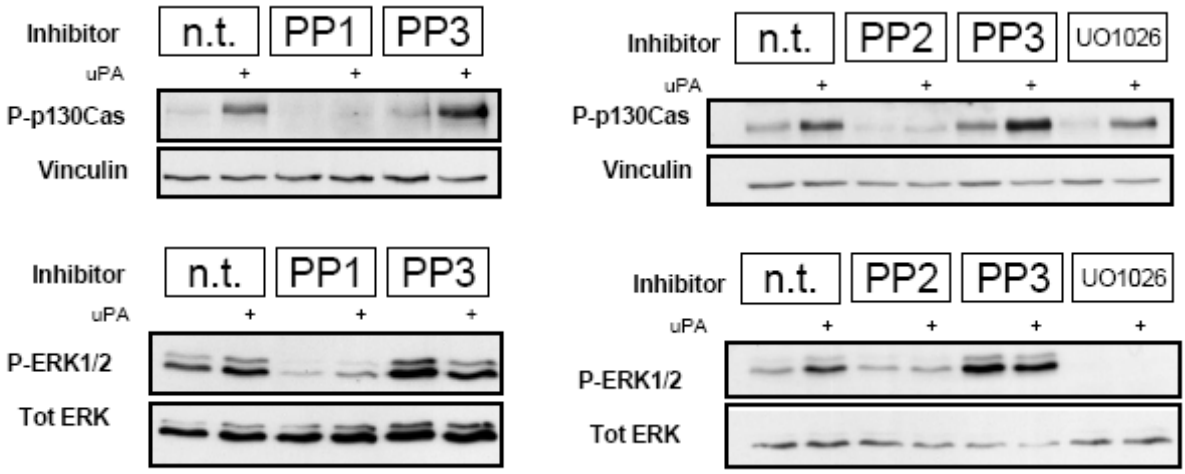


Figure 34 Ligand-independent integrin signaling to p130Cas requires SRC but not ERK activity. Western blot analysis. 293 $uPAR^{T54A}$ cells were pre-treated with Src inhibitors (PP1, PP2 and the inactive form PP3, 10 μM) or with MEK inhibitor (UO1026 20 μM) for 30 minutes and seeded on VN^{RAD} in presence or absence of uPA (10 nM). After 30 minutes cell were lysed and ERK1/2 and p130Cas phosphorylation was assayed. Representative blot are shown.

These data indicates that uPAR-VN signaling to p130Cas and ERK1/2 requires Src kinase activity. MAPK signaling pathway is not involved in p130Cas phosphorylation.

The same inhibitors were also tested for their ability to block uPAR-VN mediated cell spreading and migration on VN^{RAD}. The inhibition of p130Cas phosphorylation achieved through Src inhibitors (PP1 and PP2) was paralleled by a marked reduction of both protrusive (Fig. 35) and migratory activity (Fig.36). Inactive Src inhibitor (PP3), MEK inhibitor (UO1026) and a control inhibitor against EGFR kinase activity (AG1478) had no evident effect.

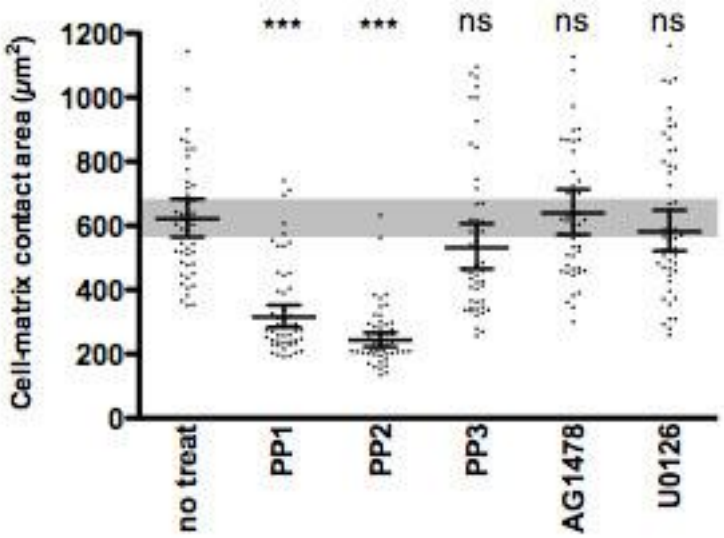


Figure 35: Src activity is required for enhancement of cell spreading downstream of uPAR-VN interaction. Quantification of cell-matrix contact area. . 293 $uPAR^{T54A}$ cells were pre-treated with Src inhibitors (PP1, PP2 and the inactive form PP3, 10 μM), with MEK inhibitor (UO1026 20 μM) or EGFR inhibitor (AG1478 250 nM) for 30 minutes and seeded on VN^{RAD} in presence or absence of uPA (10 nM). After 30 minutes cells were fixed and DIC images were acquired and quantified. Cell-matrix contact area data are means ± 95% c.i., n=50 in two independent experiments.

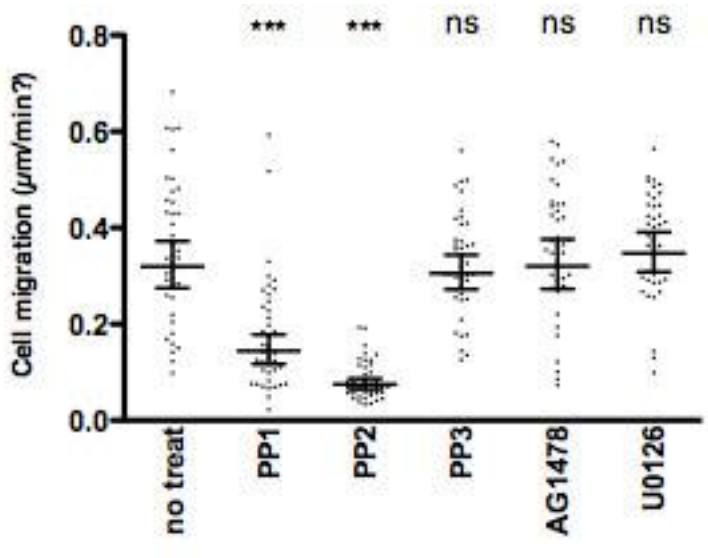


Figure 36: Src activity is required for cell migration downstream of uPAR-VN interaction. Quantification of migration speed. 293 $uPAR^{T54A}$ cells, pre-treated with Src inhibitors (PP1, PP2 and the inactive form PP3, 10 µM), with MEK inhibitor (UO1026 20 µM) or EGFR inhibitor (AG1478 250 nM) for 30 minutes and seeded on VN^{RAD} in presence or absence of uPA (10 nM). After 1 hours time lapse recordings were started and cell migration speed was quantified. Data are expressed as means ± 95% c.i., n=50 in two independent experiments

Altogether, these results suggest that uPAR-mediated cell adhesion induces ligand–independent β_1 integrin resulting in cell migration and spreading in a process that requires Src kinase activity. The MAPK activation seems to be a downstream consequence having no impact on cell morphology or motility.

DISCUSSION

uPAR-VN interaction regulates cell adhesion, spreading signaling:

The urokinase plasminogen activator receptor has been extensively involved in the processes of cell adhesion, spreading and migration through the modulation of integrin signaling (Madsen et al., 2007), (Smith et al., 2008), (Kjoller and Hall, 2001). In particular, the pathway from uPAR to Rac1 activation, responsible for enhanced spreading and cell migration, has been recently clarified. Briefly, uPAR transduces a signal through integrins that results in p130Cas SD phosphorylation and the subsequent formation of a complex with CRK and DOCK180. DOCK180 is a well known GEF involved in Rac activation, which in turn controls ruffling and lamellipodia activity leading to cell migration and invasion (Smith et al., 2008). Our previous and present data, together with other evidences (Kjoller and Hall, 2001) indicate that the uPAR capability of inducing adhesion to VN is the key point in this process. Consistently the uPAR-dependent adhesive properties of cells expressing either WT or $uPAR^{T54A}$ mutant correlates with increase in cell spreading and intracellular signaling.

Signaling downstream of uPAR in 293 cells has been extensively investigated in conditions of over-expression (Madsen et al., 2007), however in this context the use of $uPAR^{T54A}$ provides a number of major advantages. Firstly, the absence of baseline VN-binding disrupts the constitutive signaling activity of uPAR, which may have complex secondary cellular consequences such as changes in gene expression and even in complete

cell behavior reprogramming like epithelial-mesenchymal transitions (Zhang et al., 2003). Secondly, the uPA-dependence of changes in cell adhesion and signaling well replicates the majority of the described physiological activities of both uPA and uPAR observed in cell lines expressing endogenous levels of receptor (Yebra et al., 1999). Finally, the inducible nature of this system provides a potent tool for the accurate analysis of the hierarchy and kinetics of uPA/uPAR-signaling. Importantly the uPA effects on signaling and cell morphology are exclusively related to the rescue of VN binding in T54A mutant, as it is not affecting mock transfected cells or uPAR expressing cells plated on a substrate on which it cannot mediate cell adhesion.

The uPAR-VN interaction triggers RGD dependent and independent integrin signalling

Our previous and current data indicate that uPAR-induced VN adhesion facilitates integrin-matrix interaction, enhancing integrin outside-in signaling (Madsen et al., 2007). Indeed when cells are plated on an "integrin permissive" VN variant, uPAR signals both through $\alpha_V\beta_5$ and β_1 integrin. $\alpha_V\beta_5$ integrin is the endogenous integrin receptor for VN in 293 cells and its increased signaling activity could be accounted for the enhanced RGD-dependent cell spreading observed in our work. In fact $\alpha_V\beta_5$ integrin contribution in uPAR-VN signaling on a RGD-mutated VN variant (VN^{RAD}) is lost, being almost totally dependent on β_1 integrin. uPAR could reinforce the weak integrin-dependent cell adhesion to VN in 293 cells, inducing a mechanical distortion in the architecture and geometry of preformed integrin cytoplasmic complexes, leading to mechanotransduction. Indeed many reports argue that mechanical stretch of cells can lead to the partial unfolding of proteins bound to the integrin tails. This event could unmask phosphorylation sites or domain

involved in protein interaction giving rise to signal transduction (del Rio et al., 2009) (Sawada et al., 2006).

However, besides $\alpha_V\beta_5$ integrin signaling, uPAR/VN interaction signals also through β_1 integrin in a ligand-independent fashion. In fact, uPAR transmit a β_1 integrin-dependent signaling on an integrin-refractory VN substrate, enhancing cell spreading, signaling and migration. Importantly the inhibition of β_1 integrin does not affect integrin-mediated 293 cell adhesion to VN, indicating that this integrin is not involved in cell adhesion to this substrate. The comparable activation of the signaling molecules and cell spreading in cells seeded on VN^{WT} and VN^{RAD} suggest that the main signaling pathway downstream of uPAR/VN occurs independently of integrin binding to VN. The virtually complete inhibition of uPAR/VN^{RAD}-induced biological effects in 293 cells by inhibition of β_1-function antibodies might suggest that in this cell line β_1 integrins are unique in their capacity to transduce ligand-independent adhesion signaling. However, as β_1 integrin is the most abundant integrin β subunit expressed by the 293 cells as evaluated by semi-quantitative methods like FACS and microarray analysis (data not shown), the data do not allow us to exclude that also other integrins have the same capacity. In fact uPAR has been extensively described to functionally cooperate with a variety of β-integrins including β_2 ($\alpha_M\beta_2$, (Simon et al., 1996)), β_3 ($\alpha_V\beta_3$, (Smith et al., 2008)) and β_5 ($\alpha_V\beta_5$, (Franco et al., 2006)).

The uPAR-VN signaling through β_1 integrin occurs on a VN variant that cannot be engaged by integrins and induces cell spreading, presumably through the p130Cas-Rac1 axis. Surprisingly, even in the absence of integrin mediated cell adhesion, uPAR-VN interaction supports cell migration. uPAR-induced cell motility is again dependent on the same β_1 integrin signaling required for enhancement of cell spreading. However, the mechanism of uPAR adhesion and release of the adhesion contacts during cell migration is

still unknown. Indeed uPAR lacks a cytoplasmic domain, meaning that its adhesive activity cannot be tightly regulated by modulation of intracellular components, like in the case of integrins. Importantly, the affinity of uPAR for VN can be modulated by its dimerization and lipid raft partition (Cunningham et al., 2003) (Sidenius et al., 2002). The uPAR oligomerization could be modulated during cell migration in order to mediate firm cell adhesion in the dimeric/high affinity state and adhesion release in the low-affinity/monomeric state.

Ligand-independent integrin signaling induces robust p130Cas and MAPK phosphorylation. Moreover chemical inhibition experiments indicate that uPAR-mediated signaling, spreading and migration through β_1 integrin require Src kinase activity. Src is a major player in integrin signal transduction and it is crucially involved in integrin-mediated Rho GTPases regulation. Our experiments indicate that Src is involved in ligand-independent integrin signaling, but whether Src is upstream or downstream of integrins is still unclear. Several evidences in the literature implicate Src in outside-in signaling, locating it downstream of integrin. However a potential role upstream of integrin cannot be excluded. Further experiments aimed to measure integrin activation and analyze the protein complexes on integrin cytoplasmic tails upon Src inhibition will be performed in order to clarify this point.

In many cell systems cell migration is dependent on MAPK activity. However, in our cell system, MAPK inhibition does not impair p130Cas phosphorylation, cell spreading and cell migration. Importantly MAPK activation requires Src activity. This signaling pathway could have a role in processes that we did not investigate in this work, like cell proliferation and cell survival.

A new paradigm for adhesion signaling:

Our findings shed light on a new type of adhesive signaling characterized by the presence two different receptor types with distinct functions: on one side a signaling-incompetent anchoring receptor enables strong and sustained cell adhesion to the ECM, on the other side an integrin-signaling receptor "senses" the anchoring receptor-mediated cell adhesion and signals despite the missing interaction with the ECM. In a canonical outside-in signaling the two receptors mentioned above coincide, while in this novel adhesive signaling the identity between signaling and anchoring receptor is lost. (Fig. 1)

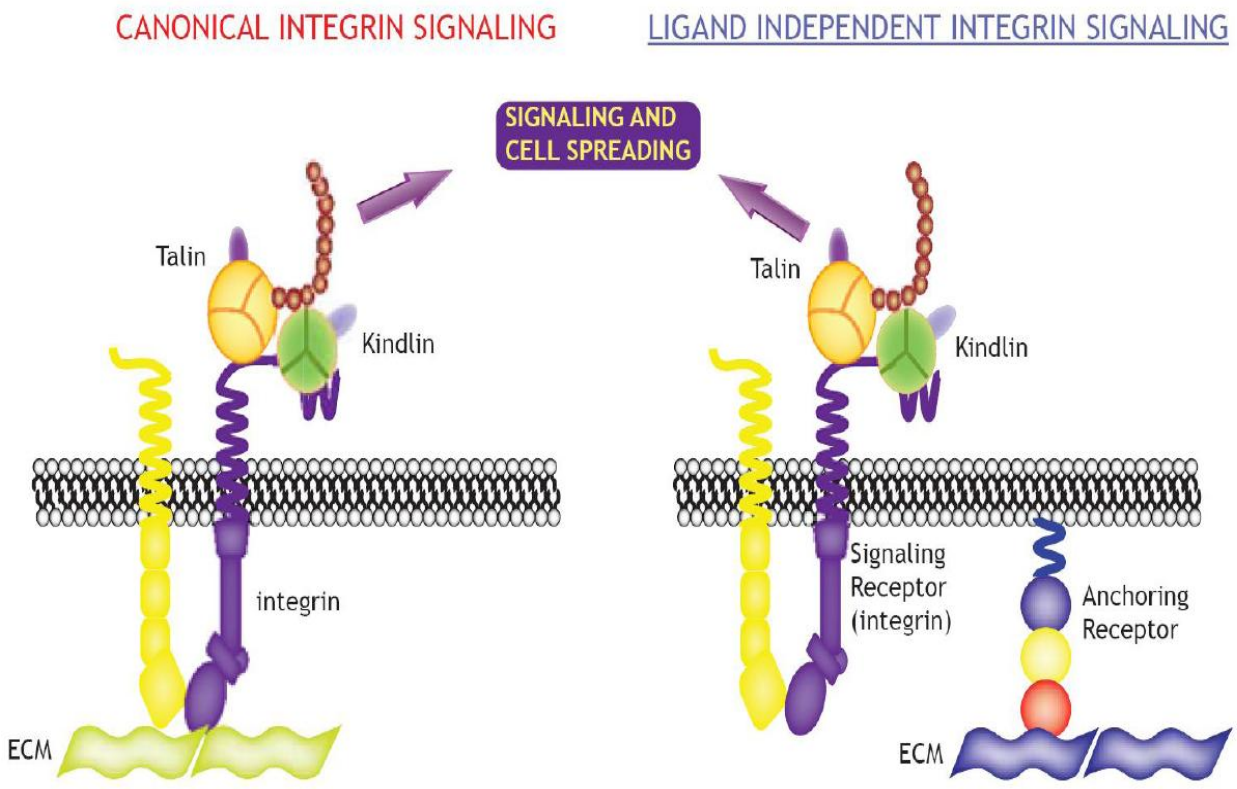


Figure 1: Schematic illustration of canonical integrin outside-in signaling (left) and ligand-independent integrin signaling. Anchoring receptor indicates a general receptor mediating mechanical cell adhesion, summarizing the features of all the different receptors used in this study.

The anchoring receptor is a signaling incompetent molecule by itself, lacking a cytoplasmic domain (uPAR and PAI-1 GPI-anchored) or the key interaction needed for signal transduction (β_3 integrin with mutation both NxxY motifs). It enables strong cell adhesion without requiring any particular membrane microdomains partition or direct interactions with the signaling receptor. Indeed the preferential partition between the soluble and insoluble fraction of the plasma membrane, manipulated by substituting uPAR-GPI anchor with transmembrane domains or different types of GPI-anchors, is not affecting uPAR adhesive function and so its ability in inducing ligand-independent integrin signaling. Even an artificial VN receptor, that shares no similarity with uPAR except the high affinity for the SMB domain of VN, recapitulates uPAR-anchoring receptor function, excluding preferential ternary structures and putative lateral interactions with the integrin-signaling receptor.

Moreover the extracellular matrix is not playing a crucial role neither as long as its components have high affinity for the anchoring receptor. Indeed VN^{RAD} can be efficiently substituted by anti-uPAR antibody coating. However, the coating components have to be immobilized on a rigid substrate in order to give rise to ligand-independent integrin signaling. In fact, either soluble anti-uPAR antibody or VN^{RAD} are not producing any effect on signal transduction when added to cells in suspension. Thus the mere interaction between the anchoring-receptor and its ligand is not sufficient to induce signal transduction but cell adhesion to a rigid substrate has to be generated. The anchoring-receptor, by mediating cell adhesion, could transit mechanical forces to the cells, thus requiring the ligand to be immobilized.

The anchoring-receptor mediated cell adhesion, besides inducing ligand-independent integrin signaling, allows the physical interaction with the matrix necessary for cell spreading and cell migration. uPAR, being a GPI-anchor protein, lacks a

cytoplasmic portion required, like in integrins, for the tight regulation of the adhesives (Franco et al., 2004). However mechanically induced cell adhesion, supported by uPAR or generally by an anchoring receptor, is sufficient to sustain cell spreading and lamellipodia protrusions even in absence of integrin-mediated adhesion. Anchoring receptor cell adhesion could be regulated by specific endocytic routes that modulates internalization of either GPI-anchored or transmembrane receptors.

Structural requirements to the signaling receptor

In our study we identified β_1 integrin as the main signaling receptor in 293 cells. Indeed the blockage of β_1 integrin function, achieved through allosteric inhibitory antibodies mAb13 and 4B4, blunted the adhesive signaling from all anchoring receptor types. These particular antibodies, rather than blocking directly integrin binding site, stabilize the extracellular portion in a close/inactive conformation (Luo et al., 2004b), suggesting the integrin active state is needed in ligand-independent adhesive signaling. This could indicate the existence of a signaling specifically and exclusively mediated by integrin activation that differs from the canonical outside-in as it does not require ligand binding.

The structure function analysis of β_1 integrin in the context of canonical outside-in and ligand-independent integrin signaling highlights the difference between these two distinct processes. The alanine substitution of Asp130 in β_1 integrin is reported to have dramatic effects on integrin function by compromising the capability of the MIDAS (metal ion dependent adhesion site) to coordinate metal ion in the integrin-matrix binding site. This produces an adhesion receptor unable to interact with its specific extracellular ligands and sustain cell adhesion without interfering with its targeting to focal adhesions (Takada et al., 1992). Consistently, D130A integrin mutant has deleterious effects on canonical

integrin-mediated adhesion and signaling. However when cell adhesion is induced by a non-integrin anchoring receptor (uPAR-VN interaction), this adhesion-incompetent mutant behaves like a functional integrin, inducing cell spreading and signaling. This could suggests that, despite the inability to engage the ECM, this mutated integrin receptor is still able to bind intracellular interactors and transduce a signaling. Moreover D130A mutant is still sensitive to mAb13 inhibition, suggesting that integrin extracellular portion structure is linked to the intracellular one, as the signaling downstream of integrin activation cannot be transmitted if the extracellular part is kept in a closed conformation by allosteric inhibitory-antibodies.

The membrane proximal and distal NxxY motifs in β_1 integrin tail, responsible respectively for talin and kindlin binding (Moser et al., 2009b), play a crucial role in both canonical and ligand-independent integrin signaling. Single (β_1 763A and β_1 775A) and combined (β_1 763/775A) alanine substitutions strongly impair integrin activation that results in the loss of adhesiveness and signaling capability (Czuchra et al., 2006). Even when the cell adhesion is rescued by uPAR/VN^{RAD} interaction, talin and kindlin deficient integrin mutants fail in transmitting the ligand-independent adhesive signaling. Moreover phenylalanine substitution (β_1 763/775F) is not affecting canonical outside-in and ligand-independent signaling, ruling out a possible role of integrin phosphorylation in these processes. Indeed, under physiological conditions phosphorylation of β_1 integrin does not seem to be required as phenylalanine substitution results in no obvious effects on cell adhesion, signaling and migration (Czuchra et al., 2006).

Even if the NPXY motifs mutations produce similar effects in both canonical and ligand-independent integrin signaling, the mechanism responsible for this effect may be different. Canonical inside-out signaling underlines the role of kindlin and talin in integrin-mediated cell adhesion. Indeed activation deficient integrins cannot engage the extracellular matrix with consequent lacking of signal transduction. On the other hand

ligand-independent adhesive signaling highlights the crucial role of talin and kindlin in signal transduction. Indeed in this process integrin-mediated cell adhesion is dispensable while their signaling capability is fully required. This is in agreement with other findings where the signaling function of talin and kindlin was assessed. Down-regulation of talin with SiRNA results in impaired FAK phosphorylation and cell spreading (Zhang et al., 2008). Manganese treatment of kindlin knock-out cells restores cell adhesion but not focal adhesion formation and cell spreading (Montanez et al., 2008). Moreover alanine substitutions in membrane proximal and distal NxxY motif in β_1 integrin result in absent focal adhesion structures and impaired cell spreading (Czuchra et al., 2006). Our data indicates that these two proteins have a crucial role in building up the basic signaling scaffold on the integrin tails inducing an early signaling by integrins in the active state, which possibly precedes the outside-in signaling by ECM engagement. However, other proteins with enzymatic activity have to be recruited and further studies will help in characterizing the nature and the composition of the protein complexes forming on integrin tails during ligand-independent adhesive signaling in comparison with canonical outside-in signaling. Moreover the different integrin conformations that occur during integrin activation (see introduction) could correspond to different signaling capabilities. Our data suggest that D130A mutant, despite the missing interaction with the ECM, retains signaling activity. The signaling capability of this integrin mutant is linked to its activation state as it is blocked by mAb13 treatment. Although D130A activity state cannot be assayed by measuring ligand binding, the analysis of this mutant in the context of ligand independent integrin signaling suggests that it has to get into an open/active conformation in order to transmit a signal. However, we cannot conclude that the activation state of D130A mutant is the same of WT β_1 integrin. For instance metal ion coordination is crucial for the right positioning of the I domain that, in the latest step of the activation process, creates the ligand binding in I domain containing α subunits (see introduction).

Our data indicate that the activation state of D130A mutant could be sufficient to induce p130Cas phosphorylation and cell spreading. Nevertheless different activation states could be paralleled by different integrin signaling capacity. For this reason further experiments aimed to exhaustively analyze the signaling and the functionality of D130A mutant in relation to its activation state are needed.

Interestingly the flexibility of the anchoring receptor type, and especially the lack of evidence supporting a direct interaction with the integrin-signaling receptor, suggest this mechanism to be more general rather than specifically related to a particular type of integrin.

As a matter of fact, β_3 integrin expression rescues the effect of β_1 integrin inhibition, proving β_3 integrin proficiency in transducing ligand-independent adhesive signaling. Moreover β_3 integrin has similar structural requirement to the ones found for β_1 integrin subunit, displaying independence of ligand binding. As for β_1 integrin, alanine substitutions in both NxxY motifs results in the loss of integrin signaling capability. Thus distinct β subunits, possibly all, can function as signaling-receptor in ligand-independent adhesive signaling when properly expressed, indicating a general mechanism. The increased vitronectin adhesion observed in β_3 integrin expressing cells indicates it to couple with endogenously expressed α_v subunit. Thus in the case of β_3 integrin, the signaling downstream of uPAR-VN interaction is transmitted by $\alpha_v\beta_3$ integrin. We have not identified the α subunit that couples with β_1 integrin, responsible for ligand-independent adhesive signaling. The fact that β_1 integrin blocking does not alter basal VN adhesion rules out the $\alpha_v\beta_1$ heterodimer. On the other hand, the elevated level of expression of α_5 subunit in 293 cells could indicate a possible involvement of $\alpha_5\beta_1$ heterodimer.

The signaling complexes on the different integrin tails could differ according to the particular heterodimer and further experiment will be aimed to characterize them. However, even if integrin-signaling receptors change, talin and kindlin interactions remain the crucial requirements in ligand-independent adhesive signaling. Moreover different integrin heterodimers activate, maybe with different modalities, p130Cas and induce cell spreading. We focused on p130Cas phosphorylation and MAPK activation because of their important role in cell migration and proliferation, respectively. However the signaling downstream of different integrin receptors could differ in terms of activation of other enzymatic activities, for instance FAK and Src family kinases. Further experiments will be needed in order to unveil and characterize exhaustively the signaling pathway(s) activate by integrin in ligand-independent adhesive signaling and compare them with the canonical inside-out signaling.

Mechanism of ligand-independent integrin signaling

One fundamental feature, required in this process, is the ability of the integrin to sense a wide variety of mechanical stimulations as ECM rigidity, topography, anisotropy and even its deformation (Geiger et al., 2009). Moreover focal adhesion components enable the cells to react to internally generated or externally applied forces. In this process the anchoring-receptor mediated cell adhesion could be considered as a force applied to the cells. This force could be sensed by integrins, or by component of focal adhesion, and translated into a biological response. Indeed mechanical stretch can alter the geometry and the folding of a vast variety of focal adhesion proteins. Talin rod has been shown to be sensitive to applied forces, unmasking cryptic binding sites for vinculin (del Rio et al., 2009). p130Cas, upon stretching, exposes the central substrate domain allowing phosphorylation (Sawada et al., 2006). Since in our system integrins are not engaging the matrix, a common

mechanotransduction mechanism cannot be responsible for ligand-independent integrin signaling. Indeed integrins are dependent on the direct contact with ECM in order to be sensitive to applied forces. However the anchoring-receptor induced cell adhesion could exploit as signaling receptors a subpopulation of integrins that are active even when cells are in suspension. It is known that platelets have to be treated with GPCR agonists in order to induce talin mediated integrin activation that enables fibrinogen binding (Han et al., 2006). However, other cell types adhere spontaneously through integrins to ECM components, without requiring any particular intracellular signaling from GPCRs or other receptors. Consistently, even in absence of specific ligands known to induce integrin inside-out signaling, 293 cells adhere to both FN and VN. Thus, integrins could be in a dynamic balance between the active and inactive state even when the cells are in suspension. The active integrin fraction could be responsible for a first contact with the ECM, which will be reinforced by ligand-induced integrin activation. These integrins could establish basic intracellular complexes on their intracellular regions containing talin and kindlin, which are needed for their active state, and therefore a primal connection with actin cytoskeleton. The force derived from mechanically-induced cell adhesion could be transmitted to these complexes, allowing other proteins with enzymatic activity to be recruited and activated, giving rise to a signal transduction event. In this view, allosteric inhibitory antibodies or mutants in the NPXY motifs would shift the integrin activation balance toward the inactive state, eliminating the integrin sub-population responsible for signaling. Moreover mechanical forces applied to the integrin β subunit can facilitate the transition from the inactive to the active conformation (Puklin-Faucher et al., 2006). Anchoring-receptor induced cell adhesion could, in this way, induce the transition towards the active integrin state. However, our experiments allow us only to conclude that an active integrin conformation is crucial in ligand-independent integrin signaling. Further experiments will be needed to test whether anchoring-receptor mediated cell adhesion

alters the integrin activation state. Antibodies recognizing the different epitopes that are unmasked during the integrin activation process could be employed to monitor the integrin conformations that characterize ligand-independent integrin signaling. Both the mechanical stimulation of primed integrins and the *de novo* integrin activation could trigger the recruitment of proteins to the integrin cytoplasmic tail, leading to the formation of a signaling complex. Interestingly integrins can sense also forces that do not directly act on FAs complexes. These stimuli encompass osmotic forces, increase in hydrostatic pressure and enhanced cell contractility. The increase in extracellular pressure activates integrin, even if the cells do not adhere to ECM (Craig et al., 2007). Mechanical cell adhesion and extracellular pressure could be considered as forces that compress the cells. Integrins could sense these forces and modify their activation state in order to allow cell adaptation to eventual changes within the extracellular environment. However, the mechanisms behind pressure-mediated integrin activation are still unknown.

Our data suggest the presence of intermediate signaling molecules between the anchoring and the signaling receptors. This would imply the existence of a "sensor" able to perceive mechanical cell adhesion and transmit it to integrins. Mechanical cell adhesion could cause the opening of stretch-activate ion channels with consequent entry of second messengers into cells. Additionally anchoring-receptor mechanical cell adhesion could act on proteins attached to the inner layer of plasma membrane like caveolin, Src family kinase and Rho GTPases altering their localization or activity. Finally anchoring receptor mediated cell adhesion could mimic integrin in causing lipid raft recycling to the plasma membrane, removing the adhesion-block and allowing signal transduction (see introduction).

Evidences supporting the notion of ligand-independent integrin signaling: new perspective for adhesion signaling

The capability of integrin in transmitting a signal without engaging their specific ECM ligands could be view as a non-intuitive, intriguing phenomenon. However, several evidences in the literature indicate that besides the canonical ligand-mediated outside-in signaling, integrins can also signal in an unligated way.

A study shows that spontaneous integrin activation, achieved through disruption of the salt bridge in the membrane proximal region, results in a constitutive intracellular signaling to FAK even when cells are in suspension. Moreover integrins carrying activating mutations and mutations in the ligand binding sites contemporaneously are targeted to focal adhesions (Hughes et al., 1996). FAK phosphorylation and integrin recruitment into focal adhesions are downstream consequences of canonical outside-in signaling. These evidences demonstrate that integrin activation can reproduce these effects independently of ligand binding. Moreover, the replacement of residues in the integrin TM domain with residues carrying a polar side chain results in increased integrin activation and favors integrin oligomerization/clustering. Interestingly cells expressing these integrin mutants display a constitutive FAK phosphorylation even when they are kept in suspension and in the absence of integrin ligands (Li et al., 2003). Thus integrin activation can initiate intracellular signaling in absence of integrin cognate ligands. However, in all of these studies integrin activation is triggered by particular mutations, while our results demonstrates for the first time that ligand-independent integrin signaling, downstream of integrin activation, is triggered by the functional crosstalk between different adhesion receptors.

The property of integrin in transducing a signal without contacting the ECM has also been shown in drosophila. Indeed integrins regulate the expression of target genes essential for embryo development. However an adhesion-incompetent chimeric protein consisting of an oligomeric extracellular domain fused to integrin cytoplasmic domain can substitute the endogenous integrin in regulating gene expression (Martin-Bermudo and Brown, 1999). This chimeric integrin variant lacks the extracellular portion, replaced by an integrin-unrelated one, but retains biological activity. This elegant study managed to uncouple integrin adhesive from integrin signaling function and demonstrates that integrin-dependent regulation of gene expression occurs also in absence of ligand binding. This is a relevant evidence demonstrating that ligand-independent integrin signaling occurs in vivo and is physiologically relevant.

The biological relevance of ligand-independent integrin signaling was also proven by another study where $\alpha_v\beta_3$ integrin was over-expressed in different cancer cell lines. Surprisingly this study revealed an unexpected function of integrins in anchorage-independent tumor growth. In particular, in cells expressing $\alpha_v\beta_3$ integrin Src associates with integrin tails even in non-adherent cells. This association results in Src activation leading to an intracellular signaling responsible for cell proliferation, also when integrins do not engage the ECM. Consistently a ligand-binding deficient integrin mutant retained its ability to induce anchorage-independent tumor growth. This study demonstrates that integrin can form an oncogenic signaling unit independently of their adhesive function also in pathological conditions like cancer (Desgrosellier et al., 2009).

These evidences together with our data strongly support the notion of ligand-independent integrin signaling. However, the biological meaning of this phenomenon is still unclear. In processes like leukocyte extravasation or tumor cell adhesion to inner vessel walls the initial cell adhesion could be induced by non-integrin adhesion molecules. This early adhesion will induce integrin activation or will allow integrin in a primed state

to signal, triggering initial cell protrusion in a ligand-independent way. Our data strongly support this hypothesis since we found out that several different ways of inducing integrin-independent cell adhesion triggers an adhesive signaling that requires integrin activation but not ligand binding. Beside the non-integrin adhesion receptors mentioned in the introduction a wide variety of other membrane receptor could have a role in cell adhesion. Moreover anchoring receptor mediated cell adhesion could be supported also by molecules that mediate cell-to-cell adhesion, thus increasing the number of potential non-integrin adhesion molecules. The anchoring receptor induces mechanical cell adhesion, therefore it does not require complex protein interactions and to undergo radical changes in its extracellular portion to mediate cell attachment. Thus non-integrin cell adhesion is a fast and undemanding way to mediate an initial contact between cells and the ECM. Interestingly, another elegant study demonstrates that actin polymerization positions activated integrin at the very front of cell protrusion like lamellipodia or filopodia (Galbraith et al., 2007). Importantly, integrins that localize at the leading edge are in an active but unligated conformation. In this scenario ligand-independent integrin signaling could trigger an initial cell protrusion phase that will facilitate active integrins to probe the matrix. This process could have a role in conditions of unfavorable ECM density and topology, where the adhesion sites on the ECM are not so easily and directly accessible. In this way the initial cell protrusion will place activated integrins in a favored position to mediate ligand binding. Finally integrin ligation will increase the early ligand-independent integrin signaling allowing proper cell polarization and migration.

The ligand-independent adhesive signaling occurs also when the anchoring and the signaling receptors are two different integrin types. Indeed mechanical cell adhesion supported by a signaling incompetent β_3 integrin transmits a signal through β_1 integrin. This could indicate that the integrin signaling is not always (or not only) transduced by the receptor that is engaging the matrix. This is not excluding the canonical outside-in

signaling to occur as β_3 integrins can signal directly without cross-talking with other integrin types. However mechanical integrin adhesion recapitulates the anchoring-receptor function proving integrins to be functional both as anchoring and signaling receptor. In physiological conditions integrins could engage the specific ligands they find in the ECM transducing directly an outside-in signaling. Moreover, since integrin mediated cell adhesion can transmit a signal through other unbound integrins, this would reinforce the whole signal transduction event, possibly by giving rise to different biological outputs. Indeed different integrins types can interact with specific ECM components, triggering different kinds of signaling that differentially regulate RHO GTPases activity (see introduction). In this way the integrin-anchoring receptor function could induce also signaling from other integrin that, for a matter of ligand specificity, cannot engage the ECM. This would amplify the integrin-mediated signal transduction repertoire, allowing a more complex and regulated biological response.

Future perspectives

In this study we identify novel mechanism of ligand-independent integrin signaling. The independence of integrin-ligand interaction is an unintuitive feature of this process. However, our data together with other evidences in the literature strongly support this alternative kind of adhesive signaling. The exact mechanism, that regulates the functional cross-talk between anchoring and signaling receptors, is still unclear. Nevertheless, understanding the mechanism of ligand-independent integrin signaling could shed new light on integrin biology and, more generally, on the modality of cell spreading and migration.

Adhesion molecules display redundancy in ligand-binding specificity and non-integrin adhesion receptors synergistically cooperate with integrin in signal transduction. Thus ligand-independent integrin signaling could be easily masked by the main canonical integrin signaling pathways. However, in light of our findings, the careful analysis of the whole adhesive signaling in complex multi-ligand conditions could lead to unexpected results. Up to now, the role of non-integrin cell adhesion in cell signaling and motility is highlighted by overexpression studies or by studies in conditions in which integrin binding is blunted. In order to address the physiological relevance of this process, models of limited or selective integrin availability combined with the manipulation of specific ECM proteins will be required. These approaches will clarify how different adhesion receptors, expressed by the same cell, contribute individually to cell adhesion and signaling in multifaceted ECM contexts. The analysis of the several steps of cell spreading and migration, from the initial phases of cell spreading and polarization to the onset of cell migration, could highlight the contribution of non-integrin cell adhesion to these processes.

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