Integrin regulation of membrane domain trafficking and Rac targeting

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Abstract

Integrins are crucial regulators of essential cellular processes such as gene expression, cell proliferation and migration. Alteration of these processes is central to tumourigenesis. Integrin signals mediate anchorage dependence of cell growth, while growth of cancer cells is anchorage-independent. Integrins critically regulate Rho family GTPases, that are also involved in cell-cycle progression and oncogenesis. In addition to their effect on GTP loading, integrins independently control the translocation of GTP-bound Rac to the plasma membrane. This step is essential for Rac binding to effectors. Integrins increase membrane affinity for Rac, leading to RhoGDI dissociation and effector coupling locally, in the vicinity of activated/bound integrins. Integrin-regulated Rac binding sites are within CEMMs (cholesterol-enriched membrane microdomains). Integrins control Rac signalling by preventing the internalization of its binding sites in CEMMs. Integrin regulation of signalling pathways initiated in CEMMs may be important for the spatial control of cell migration and anchorage dependence of cell growth.

Introduction

The integrin family of transmembrane receptors mediates cell–cell adhesion and cell attachment to the ECM (extracellular matrix) [1]. Integrins do not possess enzymatic activity. However, activation and/or ligand binding induces integrin clustering that leads to the recruitment of multiple signalling molecules and actin filaments [1]. Integrins regulate multiple pathways, including Erk, PI3K (phosphoinositide 3-kinase), FAK, Src and small Rho GTPases that induce changes in cell polarity, cell migration, cell-cycle progression, gene expression and survival [2,3]. In addition, integrin signals are frequently required for coupling growth factor receptors to downstream effectors [3]. Alteration of these pathways leading to loss of integrin requirement explains anchorage-independent cell growth and increased cell migration and invasion, characteristics that define most metastatic cancer cells [4,5].

Small Rho GTPases regulate multiple processes, including cell migration and polarization, membrane trafficking, cell-cycle progression and gene expression [6,7]. Rho GTPases regulate these processes by interacting with effector molecules that initiate various signalling cascades. Effector molecules interact with activated and GTP-loaded Rho GTPases. GTP loading is regulated by GEFs (guanine nucleotide-exchange factors) that activate the GTPase and by GAPs (GTPase-activating proteins) that inactivate them [7]. GTPases are also regulated by RhoGDIs (where GDI stands for GDP dissociation inhibitor) that keep them soluble in the cytoplasm by shielding the geranylgeranyl moiety [8] and preventing effector binding [9]. Rac1 is a small Rho GTPase that regulates survival, gene expression, cell-cycle progression, cell migration and cell–cell adhesion [7,10]. Rac function contributes to transformation induced by oncogenes, including Ras, Bcr-Abl, v-Abl and Src [6]. Rac contains a conserved CAAX sequence at the C-terminus, which is modified by a geranylgeranyl moiety [11]. The isoprenoid moiety is inserted into the plasma membrane, allowing Rac to physically associate with the plasma membrane [8,11]. It has recently become clear that integrin-regulated localization of Rac at specific plasma membrane microdomains is critical for binding to and activation of its effector Pak (p21 activated kinase) [12,13].

Integrins regulate the coupling of Rac with its effector Pak by regulating Rac membrane targeting locally

Rac is activated by growth factors present in serum in suspended and attached cells [14]. Interestingly, Rac activation level in attached cells is higher than in suspended cells. This is due to the effect of integrins on Rac activation. Cell adhesion to fibronectin in the absence of serum induces a transient activation of Rac that is similar to growth factor-induced Rac activation in non-adherent cells. Thus Rac is activated by growth factors present in serum and by cell attachment to the ECM. Notably, both stimuli are independent and accumulative [14,15]. Although Rac GTP loading can be induced by growth factors in an integrin-independent manner, downstream signalling is strictly dependent on integrins. Pak is a Rac effector that is activated by serum in attached cells; however, it is not activated in suspended cells

Key words: anchorage-dependent growth, integrin, membrane domain, rac targeting, Rho GTPase, signalling.

Abbreviations used: CEMM, cholesterol-enriched membrane microdomain; CTxB, cholera toxin subunit B; ECM, extracellular matrix; FRET, fluorescence resonance energy transfer; GDI, GDP dissociation inhibitor; GPI, glycosylphosphatidylinositol; Pak, p21 activated kinase.

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after serum stimulation, even though Rac activity is elevated under these conditions [14]. Pak isolated from suspended cells can be activated *in vitro* by Cdc42, which indicates that there are no inherent alterations in Pak under non-adherent conditions. In addition, Pak activity is an adhesion-dependent event in the presence of constitutively activated V12–Rac. This result suggests that factors independent of Rac GTP loading regulate Rac-induced Pak activation [14]. Taken together, these series of experiments indicate that adhesion to the ECM couples Rac with its effector Pak.

Several observations strongly suggest that proper Rac membrane targeting regulates effector coupling and downstream signalling. Rac translocates to the membrane fraction after serum stimulation in adherent cells, but not in suspended cells [14]. This is also the case for V12 Rac, indicating that changes in GTP loading due to integrin-mediated adhesion to the ECM do not determine Rac membrane targeting. A point mutation in the CAAX-membrane targeting motif of V12 Rac prevents Pak activation in adherent cells [14]. Although the CAAX sequence is a major determinant in Rac membrane targeting, a polybasic region (KKRKRK) near the C-terminus contributes significantly to membrane localization of Rac [9]. In addition, membrane binding assays indicate that Rac binds with higher affinity to membranes isolated from adherent cells than from suspended cells [14]. Furthermore, forced membrane targeting of Rac in suspended cells restores Pak activity [14]. These results indicate that Rac-induced Pak activation requires membrane association of Rac. Pak and other effectors also localize to the plasma membrane in activated cells [16] and therefore, it is theoretically possible that effectors drive Rac to the membrane. However, mutations in the Pak binding sequence of Rac do not alter its membrane localization, demonstrating that Pak does not directly recruit Rac to the plasma membrane. Similarly, both mutations that abolish binding to other effectors and competition of effector binding by expression of the Rac/Cdc42 binding domain of Pak (PBD) do not alter Rac membrane localization [9]. Therefore integrin-mediated adhesion to the ECM regulates Rac translocation to membranes in a manner independent of both GTP-loading and effector binding, but dependent on the C-terminal region of Rac. Upon detachment, loss of integrin-mediated adhesion to the ECM prevents Rac from being associated with membranes [14,15]. Abassi and Vuori [17] have shown that CrkII regulates adhesion-dependent Rac membrane targeting but not GTP loading, suggesting that Rac membrane targeting may be a highly regulated process. These observations underscore the importance of Rac plasma-membrane targeting to switch on downstream signalling cascades.

The interaction of Rac and its effector Pak is increased near cell edges and locally induced by integrin stimulation [9]. Micro-injection of activated Rac (GFP-tagged V12-Rac) and Pak (Alexa-546-tagged PBD) shows that both of them distribute homogenously in both cytoplasmic and membrane regions. However, a FRET (fluorescence resonance energy transfer) signal indicating interaction between the two proteins was only observed at regions near cell edges [9]. Therefore, in spite of uniform distribution of activated-Rac and the effector domain, they only interact in cell edges, close to areas of integrin activation. As expected, a GFP–Rac effector mutant (Q61L-T35S–Rac), which is activated but deficient in effector binding, shows no increase in the FRET signal at cell edges. Local clustering of integrins induced by fibronectin-coated beads triggers integrin-mediated signals [18]. Using this method, the FRET signal induced by V12Rac, but not Q61L-T35S-Rac, was greater around beads coated with integrins, but not with CD44, a molecule that mediates integrin-independent adhesion [19]. Similar to activated Rac, wild-type Rac also induced a positive FRET signal on anti-β1 integrin- and fibronectin-coated beads. However, this FRET probe does not distinguish integrin-induced Rac activation from targeting, although results with V12-Rac solely represent targeting. Interestingly, a polybasic region and activated mutant (V12-Rac-6Q) deficient in membrane localization, induces significantly lower FRET signal than V12Rac. These results reinforce previous results [14] and show that integrins induce localized targeting of Rac to membranes allowing effector binding.

Binding between Rac and the lipid bilayer is regulated by RhoGDI, which keeps Rac soluble in the cytoplasm by shielding the isoprenoid moiety [8]. RhoGDI binds Rac in the cytosol to prevent both membrane and effector binding [9]. A prenylation deficient and activated Rac mutant (V12-Rac-SAAX), unable to bind either membranes or RhoGDI [8], does not induce a FRET signal near cell edges. Instead, it induces a high FRET signal in the central part of the cell [9]. As expected, fibronectin-coated beads do not induce FRET signal with this Rac mutant [9]. These observations support the idea that RhoGDI binding prevents Rac coupling with effectors in the cytoplasm. Integrins would locally increase the affinity of the plasma membrane for Rac, favouring RhoGDI displacement and allowing Rac effector binding in the vicinity of focal adhesions. In support of this idea, binding between Rac and RhoGDI is higher in suspended than in adherent cells [9].

The effect of integrin signalling on GTPase membrane targeting is not exclusive to Rac. The small Rho GTPase Cdc42 is also translocated to the plasma membrane after agonist stimulation in an integrin-dependent manner [9]. Fractionation studies have shown that Cdc42 is enriched in the particulate fraction in adherent cells, but not in suspended cells. Furthermore, GFP-tagged Cdc42 localizes to membranes after agonist stimulation, mostly in the basal membrane, where integrins are occupied. As occurs with V12-Rac, constitutively activated Cdc42 does not localize to membranes in suspended cells [9], confirming that integrin engagement regulates membrane translocation independent of GTP loading. Recent studies [20] suggest that integrins could also affect the subcellular targeting of Rho.

In summary, these studies showed that integrins, in addition to regulating GTP loading [14,21–23], independently regulate GTP-Rac translocation to the plasma membrane allowing effector binding. GTP-Rac binds better to membranes from adherent cells than from suspended cells, suggesting

that integrins regulate Rac membrane binding sites. These studies did not identify integrin-controlled Rac-binding sites, although others had proposed Rac and Rho to be concentrated in lipid domains in the plasma membrane [24,25].

CEMMs (cholesterol-enriched membrane microdomains)

Recent models of the plasma membrane predict an organization of the cell surface into heterogeneous lipid-based microdomains co-ordinating a number of cellular functions, including signalling and membrane trafficking [26–28]. These domains are collectively referred to as 'lipid rafts' and are envisaged as highly ordered, low density, cholesterol-rich regions [29]. These domains are also enriched in sphingolipids, including gangliosides such as G_{M1} and lipid-modified proteins such as caveolins, flotillins, Src-family kinases and GPI (glycosylphosphatidylinositol)-linked proteins [26–28]. The term 'rafts' is a broad one and probably covers many different types of lipid-based membrane microdomains [30–32] that are still poorly defined [28,33,34]. There is significant uncertainty in understanding the basis for the formation of these microdomains in cell membranes and in estimating their size, density, composition and stability [33,35–38]. Indeed, visualization of native raft domains in living cells has been difficult, questioning their very existence [34]. Clearly, the study of this complex biological problem requires a combination of approaches comprising both model membrane systems and potent imaging techniques applied to membranes in living cells. Promising progress is being made recently by using fluorescence polarization anisotropy [37,39], single-particle tracking [35], FRET [36,40] and quantitative immunoelectron microscopy [38] among other techniques.

Rac targeting to cholesterol-rich domains

Previous studies had proposed Rac and Rho to be concentrated in lipid rafts and caveolae [24,25], suggesting that integrins could regulate Rac targeting specifically to these domains. Chemical disruption of cholesterol domains with methyl-β-cyclodextrin does not alter activation of endogenous Rac [12], but prevents its translocation to the membrane [12,41] and the subsequent activation of Pak [12], mimicking the effects of loss of cell adhesion. Incubation of a recombinant, isoprenylated Rac/RhoGDI complex with plasma membrane fractions purified by using a detergent-free density gradient centrifugation method [42] shows that GTP-Rac binds preferentially to the low-density, cholesterol- and caveolin-enriched fractions [12]. Moreover, GTP-Rac binds specifically to liposomes prepared with an equimolar mixture of phosphatidylcholine (PC), cholesterol and sphingomyelin (Sph), which is in a liquid-ordered state similar to CEMMs [43,44]. Targeting to lipid domains in intact cells can be assessed by labelling the ganglioside G_{M1} with its natural ligand, CTxB (cholera toxin subunit B). Activated Rac shows a strong co-localization with G_{M1}, mostly at membrane cell edges [12]. Furthermore, Rac co-aggregates with G_{M1} clustered with CTxB-coated latex beads. Therefore Rac preferentially associates *in vivo* with G_{M1}-enriched domains of the plasma membrane. Consistently, studies of fluorescence polarization anisotropy have shown a front-to-back gradient of plasma membrane microviscosity due to cholesterol accumulation at the leading edge of migrating cells [39], where it also co-localizes with Rac [45]. In addition, a peptide encoding Rac1 C-terminus localizes to lipid domains and inhibits Rac localization and function, as measured by membrane ruffling [46]. Moreover, an unbiased quantitative proteomics study revealed Rac1 as a lipid domain-associated protein [47]. In summary, Rac shows a GTP-dependent selective binding to lipid domains, which, at least partially, seems to be determined by lipids themselves.

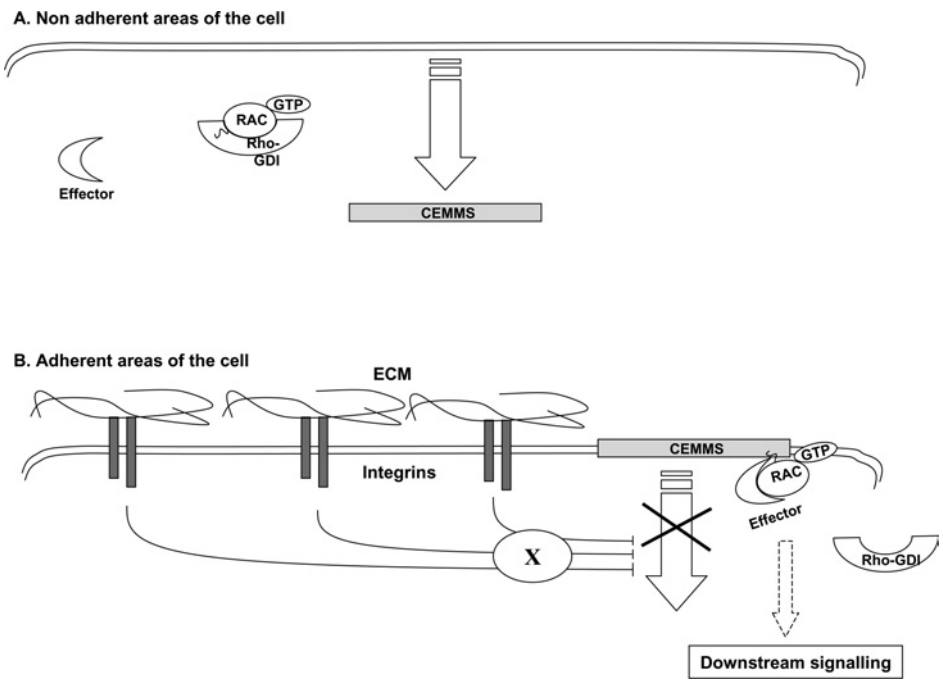
Integrin regulation of membrane domain localization

These findings led to the hypothesis that CEMMs may be the targets for integrin regulation of Rac translocation to the membrane, as these domains seem to provide the membrane binding sites for Rac. To address this question, effects of integrins on the distribution of CEMMs markers were studied. Loss of integrin-mediated adhesion causes a rapid internalization of several cholesterol-rich domain markers, including cholesterol itself, G_{M1} and GPI-anchored proteins. The effects are specifically reversed by replating the suspended cells on fibronectin or anti-β1 integrin antibodies, but not on non-integrin adhesive substrata. Consistently, local clustering of integrins with beads coated with anti-β1 antibodies promotes local accumulation of both G_{M1} and Rac [12]. Therefore internalization of CEMMs is specifically regulated by integrins. One striking question is whether Rac remains bound to CEMMs after internalization. Existing evidence shows that upon cell detachment and subsequent cholesterol-rich domain internalization, Rac dissociates from the total membrane fraction and remains bound to RhoGDI in the cytoplasm [14]. Therefore Rac and CEMMs probably dissociate after loss of integrin-mediated cell adhesion. Biochemical alterations in the composition of the internalized lipid domains could account for this observation.

These results suggest that the integrin-controlled internalization of CEMMs mediates loss of Rac membrane targeting and downstream signalling upon cell detachment. To test this hypothesis, internalization of G_{M1}-rich domains in non-adherent cells was blocked artificially by treating cells with CTxB-beads before detachment. Under this condition, non-adherent cells retain plasma membrane association of not only G_{M1} domains, but also Rac, as well as Rac activation of Pak [12]. These results could also be explained by local recruitment of integrins mediated by CTxB beads and subsequent Rac activation. However, CTxB beads neither recruit integrins nor increase Rac-GTP loading. Therefore internalization of G_{M1}-rich microdomains from the plasma membrane is required for the loss of Rac targeting and downstream signalling after detachment from the ECM.

Figure 1 | Model for integrin spatial regulation of Rac downstream signalling

Integrins regulate Rac signalling spatially by preventing the internalization of Rac binding sites within CEMMs. The cell has been artificially divided into two areas. (**A**) Regions where integrins are not occupied/activated, mimicked experimentally by cells detached from the ECM. In these areas, CEMMs undergo internalization, which prevents Rac plasma membrane localization. Thus Rac remains in the cytoplasm bound to RhoGDI and uncoupled from downstream signalling mediated by effector molecules. (**B**) Zones of the cell where integrins are occupied by ECM-ligand and/or activated. Local activation of integrin signalling through unknown mediators (X) prevents CEMMs internalization locally. Thus in plasma membrane edges near these areas, CEMMs mediate binding of activated Rac, which is able to couple with effectors and trigger downstream signalling. This mechanism could be important for the spatial control of cell migration and anchorage dependence of cell growth.



Concluding remarks and future directions

We propose a model based on all these observations (Figure 1). Rac association with the plasma membrane and activation of its downstream effectors requires membrane binding sites that are controlled by integrins. These sites are components of CEMMs. Integrin-mediated adhesion maintains membrane domains at the cell surface. When cells are detached, CEMMs are cleared from the plasma membrane through internalization, disrupting the Rac signalling pathway. Overall, the data indicate that integrins mediate Rac targeting and signalling by inhibiting internalization of Rac binding sites in cholesterol-rich domains (Figure 1).

A similar picture emerges from the observation that integrins control Rho-mediated microtubule stabilization by maintaining G_{M1}-rich domains at the cell leading edge [48]. Therefore integrins regulate targeting and signalling of at least two pathways (Rac and Rho) by their effects on CEMMs. This finding supports the proposed model of signalling regulation by integrins through internalization of specific membrane microdomains. In fact, an interesting question is whether internalization of CEMMs might be a mechanism by which integrins influence signalling pathways other than Rac and Rho. Several studies have suggested that many signal transduction pathways highly dependent on integrins are also dependent on lipid domains, such as Ras/Erk, JNK, PI3K-Akt, FAK and Src-family kinases [26,49–51]. These pathways are also implicated in cell-cycle- and anchorage-dependent growth [2,4,5]. It is tempting to speculate that inhibition of domain internalization by integrins could be a key event in anchorage-dependent cell growth. A bypass of the integrin requirement at this level could lead to anchorage-independent growth, characteristic of transformed cells. This is undoubtedly an interesting area of future research.

Other questions that remain unsolved are to elucidate the mechanism by which integrins regulate lipid-domain trafficking (although FAK has been proposed [48], others could also participate), what lipid domains are specifically involved in each pathway, what are the consequences of this mechanism in the control of directed cell migration etc. Finally, it is important to underline that most data discussed here were obtained in anchorage-dependent cells such as

fibroblasts, epithelial and endothelial cells. The fact that Rac does not seem to target to lipid domains in haematopoietic cells [52] underscores the importance of studying integrin-regulation of membrane domains in different cell types.

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