Rac1 and RhoA GTPases have antagonistic functions during N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts.

Franck Comunale, Marie Causeret, Cyril Favard, Julien Cau, Nicolas Taulet, Sophie Charrasse, Cécile Gauthier-Rouvière

HAL Id: hal-00216159
https://hal.science/hal-00216159

Submitted on 24 Jan 2008

Rac1 and RhoA GTPases have antagonistic functions during N-cadherin-dependent cell-cell

contact formation in C2C12 myoblasts

Franck Comunale[1], Marie Causeret[1], Cyril Favard[2] , Julien Cau[1], Nicolas Taulet[1], Sophie Charrasse[1], and Cécile Gauthier-Rouvière[1*]

[1]CRBM, CNRS, 1919 Route de Mende, 34293 Montpellier France

[2]IPBS, 205 Route de Narbonne, 31077 Toulouse France

*Corresponding author: cecile.gauthier@crbm.cnrs.fr

Tel: (33)467613355

Fax: (33)467521559

E-mail: cecile.gauthier@crbm.cnrs.fr

Running title : Rho GTPases in N-cadherin-based adhesion

Key words : RhoA, Rac1, N-cadherin

Abstract

Background Information: N-cadherin, a member of the Ca^{2+}-dependent cell-cell adhesion molecule family, plays an essential role in the induction of the skeletal muscle differentiation program. Yet the molecular mechanisms, which govern the formation of N-cadherin-dependent cell-cell contacts in myoblasts remain unexplored.

Results: Here we show that N-cadherin-dependent cell contact formation in myoblasts is defined by two stages. In the first phase, N-cadherin is highly mobile in the lamellipodia extensions between the contacting cells. The second stage corresponds to the formation of mature N-cadherin-dependent cell contacts, characterised by the immobilization of a pool of N-cadherin, which appears to be clustered in the interdigitated membrane structures that are membrane attachment sites for F-actin filaments as well. We also demonstrate that the formation of N-cadherin-dependent cell-cell contacts requires a coordinated and sequential activity of Rac1 and RhoA. Rac1 is involved in the first stage and facilitates N-cadherin-dependent cell-cell contact formation, but it is not absolutely required. Conversely, RhoA is necessary for N-cadherin-dependent cell contact formation, since, through ROCK signalling and Myosin 2 activation, it allows the stabilization of N-cadherin at the cell-cell contact sites.

Conclusions: We show that Rac1 and RhoA have opposite effects on N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts and act sequentially to allow its formation.

Introduction

Cadherins are transmembrane receptors that mediate calcium-dependent cell-cell adhesion. They play essential roles during embryonic development and in the maintenance of tissue architecture in adult life. They are involved in differentiation, cell growth and migration. Formation of cell-cell contacts is a multi-step process that includes cadherin association with catenins, delivery of cadherin-catenin complexes to the plasma membrane, lateral diffusion in the plasma membrane toward the cell-cell contact sites, cadherin oligomerization, homotypic interactions and cadherin complexes association with the actin cytoskeleton. Much attention has been focused on the role of cytoplasmic proteins, such as the Rho family small GTPases, in cadherin-dependent adhesion, as they might regulate this process at different steps. Indeed, the Rho GTPases are well known for their pleiotropic activities on the actin cytoskeleton, vesicular trafficking, protein phosphorylation and gene transcription (Schwartz, 2004). Members of this family of GTP-binding proteins have emerged as key mediators of cadherin activities (Fukata and Kaibuchi, 2001; Braga, 2002). Nevertheless, the involvement of each Rho family small GTPase in cell-cell adhesion differs, depending on the cellular context and the maturation status of the junction (Braga *et al.*, 1997; Kuroda *et al.*, 1997; Takaishi *et al.*, 1997; Jou and Nelson, 1998; Braga *et al.*, 1999; Yap and Kovacs, 2003). Moreover, the mechanisms that regulate the establishment of cell-cell adhesion, and the role of the Rho family small GTPases have been extensively studied in epithelial or endothelial cells, while not as many data are available for other cell types. This is the case for the skeletal muscle cells, in which cadherins play important roles at different steps of skeletal muscle biogenesis. In particular, N-cadherin is expressed early in the somites, myotome and limb buds, and is down-regulated as synaptogenesis and secondary myogenesis proceed (Duband *et al.*, 1987; Hatta *et al.*, 1987; Linask *et al.*, 1998). N-cadherin-dependent inter-cellular

adhesion has a major role in cell cycle exit and in the induction of the skeletal muscle differentiation programme (George-Weinstein et al., 1997; Goichberg & Geiger, 1998; Mege et al., 1992; Redfield et al., 1997; Knudsen, 1990; Cinnamon *et al.*, 2006). N-cadherin triggers signalling events that promote the commitment to myogenesis through RhoA positive and Rac1 negative regulation (Charrasse et al., 2002). In myoblasts, RhoA activity is required for β-catenin accumulation at cell-cell contact sites, another event involved in myogenic induction (Goichberg *et al.*, 2001; Charrasse *et al.*, 2002). These crosstalks between cadherin and Rho GTPases appear to be important both during myogenesis and during pathological development of rhabdomyosarcoma (RMS). In fact, it has been shown that in RMS-derived cell lines N-cadherin is either down-regulated or mislocalized and Rac1 is constitutively activated (Meriane *et al.*, 2002; Charrasse *et al.*, 2004).

Since Rho signalling plays an important role in the formation and/or maintenance of cadherin-dependent adhesion and this function varies according to the type of cells and cadherins studied, it becomes important to specifically analyse the role of Rac1 and RhoA in N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts. Hence, in this study, we focus on three important questions that have not yet been addressed in the literature. First, we detail the morphological organisation of the N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts. Second, we analyse Rac1 role in N-cadherin-dependent cell-cell contact formation, and finally we focus on the role of RhoA in this process. Our results reveal that in C2C12 myoblasts Rac1 and RhoA have opposite effects. Expression of an activated form of Rac1 disrupts the formation of N-cadherin-dependent cell-cell contacts, whereas activated RhoA reinforces it. In addition, we show that RhoA, but not Rac1, is absolutely required for N-cadherin-dependent cell-cell contact formation.

Results

Formation of cell-cell contacts between myoblasts

Two distinct stages have been observed during cell-cell contact formation in myoblasts: the AJ1 step, which corresponds to a developing cell-cell contact and the AJ2, relative to an already established contact (Causeret et al., 2005). To better understand the organization of the cell-cell contacts at these stages, C2C12 myoblasts were analysed for N-cadherin and F-actin distribution (Figure 1). In developing cell-cell contacts we always observed overlapping lamellipodia (panels a-c), which gradually disappear to give rise to interdigitated membranes (panels d-f). Filopodia are also visible albeit to a lesser extent (arrows in panel a', b'). N-cadherin is found at the cell-cell contacts, as early as the AJ1 stage, and is accumulated during the AJ2 stage. The AJ2 stage is characterised by the presence of actin stress fibres between the two adjacent cells at the end of which N-cadherin is found (panels d-f).

This transition from the AJ1 to AJ2 stage can be observed upon induction of calcium-dependent intercellular adhesion. Cadherins require Ca^{2+} to form homophilic cell-cell adhesions ; thus, Ca^{2+} chelation with EGTA, followed by a new addition of Ca^{2+}, offers a straightforward method to study the dynamics of cell-cell contact formation (Volberg *et al.*, 1986; Charrasse *et al.*, 2002). Control cells presented a typical pattern of N-cadherin immunostaining at the cell-cell contacts during the AJ2 stage (Figure 1B, panel a). Upon treatment with EGTA, N-cadherin was progressively lost from the cell-cell contact sites, which then resembled typical AJ1 stage cell-cell contacts (panel b). After restoration of the extracellular Ca^{2+}, the cell-cell contacts were rapidly reformed as shown by the N-cadherin staining (panels c to f) and cells progressed from the AJ1 toward the AJ2 stage (around 30 min after adding Ca^{2+}).

Antagonistic effects of Rac1 and RhoA on N-cadherin-dependent cell-cell contact formation

We analysed the effects of RhoA and Rac1, two important Rho GTPases, on N-cadherin-dependent cell contacts formation during myoblast differentiation or transformation (Meriane *et al.*, 2000; Charrasse *et al.*, 2002; Meriane *et al.*, 2002).

We first analysed the effects of the expression of constitutively active or dominant negative Rac1 mutants (GFP-Rac1V12 and GFP-Rac1N17, respectively) on the N-cadherin-dependent cell-cell contact formation (Figure 2A). Control C2C12 myoblasts, which expressed an empty pEGFP vector, presented the distinctive pattern of the AJ2 N-cadherin immunostaining at cell-cell contacts (panels a-c), whereas the expression of GFP-Rac1V12 impaired the accumulation of N-cadherin at these sites, which where thus reminiscent of the AJ1 stage (panels d-f). GFP-Rac1N17 expression had no effect on N-cadherin accumulation at the cell-cell contact sites, as shown by their characteristic AJ2 stage expression (panels g-i), similarly to what has been observed in the control cells. We next analysed the effect of the expression of these Rac1 mutants, during restoration of cell-cell adhesions after their disruption by chelation of the extracellular Ca^{2+} (Figure 2B and C). Rac1V12-expressing cells showed the typical AJ1-type contact sites (i.e. absence of N-cadherin accumulation) in untreated cells (Figure 2B, panels a, g and m), EGTA-treated cells (Figure 2B, panels b, h and n) and after addition of extracellular Ca^{2+} (Figure 2B, panels c-f, i-l and o-r). Data collected at later times after restoration of extracellular Ca^{2+} showed that Rac1V12 was not merely delaying the AJ1 to AJ2 transition, but inhibiting it (data not shown). Conversely, Rac1N17 expression did not impair the accumulation of N-cadherin at the cell-cell contacts (Figure 2C, panels), but nevertheless delayed the progression towards the AJ2 stage (compare Figure 1B to 2C).

We next analysed the effects of the expression of constitutively active or dominant negative RhoA mutants (GFP-RhoAV14 and GFP-RhoAN19, respectively) on N-cadherin-dependent cell-cell contact formation (Figure 3A). Expression of RhoAV14 increased the accumulation of N-cadherin at the cell-cell contacts (panels a-e), whereas RhoAN19 decreased its accumulation and favoured the formation of AJ1-type cell-cell contacts (panels f-j). We also examined the effects of the expression of these RhoA mutants during the restoration of cell-cell adhesion after disruption by chelation of the extracellular Ca^{2+} (Figure 3B and C). RhoAV14-expressing cells behaved like the Rac1N17-expressing ones, since no AJ1-type cell-cell contacts were observed at any time of the Ca^{2+}-switch process (Figure 3B). Again, the AJ2 stage appeared later than in control cells. However, in contrast to the Rac1N17-expressing cells, RhoAV14 expression increased N-cadherin accumulation at the cell-cell contacts above the normal level (compare Figure 3B to Figure 1B). Expression of RhoAN19, as that of Rac1V12, favoured the formation of the AJ1 stage cell-cell contacts and inhibited the AJ1 to AJ2 transition, which is normally observed during N-cadherin-dependent cell-cell contact formation.

Taken together these data demonstrated that 1) Rac1 and RhoA have opposite effects on N-cadherin-dependent cell-cell contact formation, 2) expression of an active Rac1, like that of a dominant negative RhoA, favours the formation of AJ1-type cell-cell contacts and the inhibition of the AJ1 to AJ2 transition, 3) expression of an active RhoA or of a dominant negative Rac1 impairs the appearance of the AJ1 stage and delays the AJ2, 4) active RhoA increases N-cadherin accumulation at the cell-cell contacts.

Effects of Rac1 and RhoA on N-cadherin-dependent adhesive complex stabilization at the cell-cell junctions

To understand the mechanism by which Rac1 and RhoA regulate N-cadherin accumulation at the cell-cell contact sites, we first used a biotinylation assay to analyse the presence of N-cadherin at the cell surface. We analyzed both total and cell surface biotinylated N-cadherin in control and Rac1V12, Rac1N17, RhoAV14 and RhoAN19-expressing myoblasts. The histogram shows the level of biotinylated N-cadherin normalised to the amount of total cellular N-cadherin (Figure 4A). No non-biotinylated N-cadherin binding to streptavidin beads was observed (data not shown). These figures led us to conclude that neither the activation nor the inhibition of Rac1 or RhoA modify the amount of N-cadherin at the plasma membrane and suggested that they only regulate the regional distribution of N-cadherin at the cell surface.

To analyse whether the expression of Rac1 and RhoA affects the assembly dynamics of N-cadherin at the cell-cell contacts, we performed fluorescent recovery after photobleaching (FRAP) experiments and measured the diffusion coefficient (D) and the mobile fraction (M) of N-cadherin/GFP in living C2C12 myoblasts. We first examined the N-cadherin/GFP dynamics during cell-cell contact establishment, which correspond to the transition from the AJ1 to AJ2 stage. As previously reported, we observed a decrease in the lateral diffusion and the mobile fraction of N-cadherin/GFP at the AJ2 cell-cell contacts (Figure 4B and C) (Causeret et al., 2005). The expression of RFP-Rac1V12 led to an increase in the mobile fraction and lateral mobility of N-cadherin/GFP. In contrast, expression of RFP-RhoAL63 (i.e. constitutively active RhoA) decreased the mobile fraction and lateral mobility of N-cadherin/GFP. These opposite effects of the constitutively active forms of Rac1 and RhoA are illustrated on the recovery curves (Figure 4B), the histogram (Figure 4C) and the representative N-cadherin/GFP images

immediately before photobleaching and immediately after photobleaching and at the indicated times after recovery (Figure 4D). These data suggest that the expression of active Rac1 and RhoA have opposite effects on the stability of N-cadherin at the cell-cell contacts, since Rac1 increases and RhoA decreases the lateral diffusion and mobile fraction of N-cadherin at the cell-cell contacts.

N-cadherin-dependent cell-cell contact formation is regulated by the ROCK/MLCK/Myosin 2 pathway

In order to better understand the mechanisms of N-cadherin association at the cell-cell contacts, we have tested various drugs acting on the N-cadherin localisation at these sites at steady state or using the Ca^{2+} switch protocol described in Figure 1B. As summarized on Table 1, we have been able to identify conditions, which either destabilize or stabilize N-cadherin at the cell-cell contacts in myoblasts. Particularly, inhibition of either the serine/threonine Rho-associated kinase ROCK, using Y-27632, or Myosin Light Chain Kinase (MLCK), with ML-7, or of its substrate Myosin 2, with blebbistatin, impaired N-cadherin accumulation at the cell-cell contacts (Figure 5A). These results are comparable to those obtained in Rac1V12 or RhoAN19-expressing cells (Figure 2 and 3, respectively), or after addition of an anti-N-cadherin antibody raised against its extracellular domain (Charrasse et al., 2002). None of these drugs affected the total cellular level of N-cadherin or the amount expressed at the cell surface, which were measured using a biotinylation assay (Figure 5B).

We also analysed the effect of these drugs on the assembly dynamics of N-cadherin at cell-cell contacts by FRAP. N-cadherin/GFP-expressing myoblasts, that had already established cell-cell contacts for 24-36 hours, were treated either with blebbistatin, ML7 or Y27632. This study

revealed that the rates of fluorescence recovery at the sites of cell-cell contact were increased by addition of these drugs (Figure 5 C). The measure of the N-cadherin/GFP diffusion coefficient and mobile fraction showed that the addition of any of these drugs increased both parameters (Figure 5 D).

These data indicate that the inhibition of the ROCK/MLCK/Myosin 2 pathway caused the loss of N-cadherin from the cell-cell contacts and its redistribution on the cell surface, away from these sites. This was accompanied by an increase in both N-cadherin lateral diffusion and mobile fraction at the cell-cell contacts.

RhoA promotes N-cadherin-dependent cell-cell contact formation in rhabdomyosarcoma cells

The results outlined above suggest that RhoA is a key regulator of the assembly of N-cadherin-dependent cell-cell contacts. We, therefore, decided to investigate whether RhoA could promote the accumulation of N-cadherin at the cell-cell contact sites in RD cells, a tumor cell line of skeletal muscle origin in which N-cadherin is delocalized from the cell-cell contacts (Charrasse et al., 2004). Whereas pEGFP-transfected and untransfected RD cells showed no accumulation of N-cadherin at the cell-cell contacts (Figure 6A, panels a and b), expression of GFP-RhoAV14 (panel c) increased its accumulation at the cell-cell contact sites (panel d). A quantitative analysis of these observations revealed that RhoAV14 increased the fluorescence intensity of the N-cadherin staining at cell-cell contacts (Figure 6B). Moreover, a statistical analysis showed that N-cadherin localization at the cell-cell junction is significantly induced by RhoAV14 but not Rac1N17, Rac1V12 or RhoAN19 (Figure 6C).

Discussion

Our kinetics analysis of N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts shows a spatio-temporal reorganization of plasma membranes and cortical actin. The first step corresponds to the extension of a large lamellipodium and to a lesser extent to filopodia formation. Such lamellipodia were also described in epithelial cells, such as MDCK, whereas, in keratinocytes, the filopodia initiate the cell-cell contact formation (Vasioukhin and Fuchs, 2001; Ehrlich *et al.*, 2002). In myoblasts, N-cadherin is homogeneously distributed in the plasma membrane during this first step of cell-cell contact formation in myoblasts (AJ1). By contrast, during the second step (AJ2), N-cadherin appears to be clustered in the interdigitated membrane structures, which are also membrane attachment sites for F-actin filaments (Figure 7). These clustered cadherin receptors are called puncta, plaques or adhesion zippers in epithelial cells and are characterised by interdigitation of neighbouring membranes and actin recruitment (Adams *et al.*, 1998; Vasioukhin *et al.*, 2000). In epithelial cells, but not in myoblasts, further reoganization occurs in the formation of a cortical actin ring important to generate a polarized epithelial morphology (Adams *et al.*, 1998; Zhang *et al.*, 2005). Fluorescence recovery after photobleaching (FRAP) and single particle tracking (STP) studies have shown that the formation of these structures, in which cadherins are clustered, is accompanied by the immobilisation of the cadherin receptors (Kusumi *et al.*, 1993; Adams *et al.*, 1998; Sako *et al.*, 1998; Causeret *et al.*, 2005). Amongst the different factors that regulate this stabilization process, homophilic interactions, association to the detergent-resistant membrane microdomains and a dynamic change in cadherin-cytoskeleton interactions were identified during cell-cell contact formation (Sako *et al.*, 1998; Causeret *et al.*, 2005).

11

Rho family small GTPases, well known regulators of cytoskeleton dynamics, particularly Rac1 and RhoA, are involved in the regulation of cadherin-mediated cell-cell adhesion (Fukata and Kaibuchi, 2001). However, their effects appear different regarding the type of cadherin and/or the cellular context in which the cadherin is expressed. It was, thus imperative to analyse the role of Rac1 and RhoA in N-cadherin-dependent cell-cell contact formation in myoblasts. Both Rac1/RhoA GTPases and cadherin-based cell adhesion are key regulators of the physiopathology of skeletal muscle development (Charrasse *et al.*, 2003). Skeletal myogenesis is a temporally and spacially regulated multi-step process, which involves cell-cell interactions. In particular, N-cadherin-dependent intercellular adhesion plays a major role in the induction of the skeletal muscle differentiation program (George-Weinstein *et al.*, 1997; Goichberg and Geiger, 1998; Charrasse *et al.*, 2002). In this study we have focussed on the role of Rac1 and RhoA in N-cadherin-dependent cell-cell contact formation.

A constitutively active form of Rac1 inhibited the formation of N-cadherin-dependent cell-cell contact by blocking the AJ1 to AJ2 transition. FRAP analysis showed that the active form of Rac1 increases N-cadherin diffusion at the cell-cell contacts as does the disruption of homophilic interaction, F-actin cytoskeleton or lipid rafts (Causeret et al., 2005). Rac1 is well known to induce the disassemby of actin stress fibers and the remodeling of cortical actin and might thus inhibit the anchoring of N-cadherin to the actin cytoskeleton (Ridley et al., 1992). Nevertheless, in a previous study using N-cadherin-coated beads, it has been mentioned that the active form of Rac1 did not alter their anchoring to the cytoskeleton (Lambert et al., 2002). This discrepancy might come from the analysis of different cellular compartment. Indeed, our analysis is performed on the pool of N-cadherin at the cell-cell contact whereas N-cadherin-coated beads were applied on the lamellipodia of motile myogenic C2 cells. The expression of a dominant negative Rac1 showed no effects on N-cadherin-dependent cell-cell contacts, suggesting that it

12

does not alter the structure nor destabilize the preexistent complexes. However, upon induction of cell-cell contacts formation we observed that its expression impaired the formation of the AJ1 and delayed the appearance of the AJ2 stage. On the whole, these data show that active Rac1 might be involved in the initiation of cell-cell contact through the formation of lamellipodia extensions which increases the surface area between contacting membranes and also through the increase in the lateral diffusion of N-cadherin in the contacting membranes, both events favouring cadherin homophilic interactions at this place. Thus, Rac1 activity is not required for, but facilitates, the formation of N-cadherin-dependent cell-cell contacts in myoblasts, since they form with a slower kinetic in myoblasts which express the dominant negative Rac1. A similar role for Rac1 was reported in E-cadherin-dependent cell-cell contact formation in MDCK cells or by using a reconstituted E-cadherin-dependent cell-cell adhesion assay, whereas in keratinocytes Rac1 seemed to be required for the establishment of E-cadherin-dependent cell-cell contacts (Braga *et al.*, 1997; Takaishi *et al.*, 1997; Jou and Nelson, 1998; Ehrlich *et al.*, 2002; Chu *et al.*, 2004).

In contrast to Rac1, RhoA is absolutely required for N-cadherin-dependent cell-cell contact formation in myoblasts. The initial recognition of N-cadherin at cell-cell contacts and actin recruitment at new junctions can occur in the absence of RhoA activity, but without progression toward the AJ2 stage. The constitutively active form of RhoA increases the accumulation of N-cadherin at the cell-cell contact sites along with the reinforcement of the F-actin fibres at these sites. This contrasts with the effect of RhoA on M-cadherin and again illustrates that similar perturbations of GTPase activity had a differential effect on N- or M-cadherin (Charrasse et al., 2006). FRAP analysis demonstrates that the expression of the constitutively active form of RhoA decreases the mobility and the size of mobile fraction of N-cadherin at the cell-cell contact. Nevertheless, the constitutively active form of RhoA delays the formation of the established cell-

cell contacts (AJ2) probably because it also impairs the formation of AJ1 stage. In summary, initial cell-cell contact formation (AJ1) is facilitated by Rac1 activity, whereas it is impaired by high RhoA activity. Conversely, formation of mature N-cadherin-dependent cell-cell contacts (AJ2) requires RhoA activity, but is impaired by Rac1 activation. Thus, Rac1 and RhoA have opposite effects on N-cadherin-dependent cell-cell contact formation in C2C12 myoblasts and act sequentially to allow its formation.

Since in myoblasts Rac1 activation and lamellipodia formation at the cell-cell contact sites are independent of N-cadherin activity (Charrasse *et al.*, 2002), and RhoA, but not Rac1, is required for the formation of N-cadherin-dependent cell-cell contacts (this study), we, thus, focussed our attention on the role of RhoA downstream pathways in N-cadherin-dependent cell-cell contact formation in myoblasts. We found that RhoA signalling through its effector ROCK, which activates Myosin 2 by inhibiting myosin phosphatase and also by phosphorylating myosin light chain (MLC) (Amano *et al.*, 1996; Kimura *et al.*, 1996), is required for the AJ1 to AJ2 transition. The role of Myosin 2 in N-cadherin-dependent cell-cell contact formation is also supported by its direct or indirect inhibition with blebbistatin or ML-7, respectively. We show that the RhoA/ROCK/Myosin 2 pathway exerts a positive effect on N-cadherin stabilization at the cell-cell contacts and on the AJ1 to AJ2 transition, possibly due to the role of Myosin 2 in actin bundles formation and actin contractility, which might support lateral cadherin clustering. Even though a similar positive contribution of Myosin 2 to the cadherin function has been reported (Shewan *et al.*, 2005), other studies have implicated this protein in processes that negatively affect cadherin activity (Sahai and Marshall, 2002; Avizienyte *et al.*, 2004).

Cell-cell junctions often become disrupted as tumours progress to high-grade advanced disease. Rho GTPases have been implicated in multiple steps during cellular transformation, including alterations on the adhesion status of tumor cells (Lozano *et al.*, 2003). Rhabdomyosarcomas

(RMS) are tumours of skeletal muscle origin that represent 5-10% of childhood cancers and more than 50% of the paediatric soft tissue sarcomas (Merlino and Helman, 1999). Rac1 was found constitutively activated in RMS-derived cell lines (Meriane *et al.*, 2002), where a loss of N-cadherin from the cell contacts in these cells has been described (Charrasse *et al.*, 2004). This is consistent with our data, which show that constitutive Rac1 activation decreases N-cadherin stabilization at the myoblast contact sites and inhibits AJ1 to AJ2 transition. Nevertheless, N-cadherin localization at the cell-cell contact in RMS-derived cell line was only partially restored by the expression of a dominant negative Rac1, suggesting that other pathways are involved. This is also supported by data showing that expression of an active RhoA in RMS cells does not decrease the overall Rac1 activity (data not shown). Moreover, RhoA activation, which promotes actin bundle formation and N-cadherin stabilization at the cell-cell contact sites in myoblasts, also favours N-cadherin-dependent AJ formation in a RMS-derived cell line. This is in contrast to data obtained in epithelial tumours, where RhoA/ROCK signalling destabilizes cell junctions by increasing contraction forces (Zhong *et al.*, 1997; Sahai and Marshall, 2002), and again emphasises that we must precisely analyse the role of Rho GTPases on adherens junction formation in all different cell systems.

Acknowledgements

Figures legend

Figure 1 : Cell-cell contact formation in C2C12 myoblasts

A)   N-cadherin (panels a, a', d and d') and F-actin (panels b, b', e and e') distribution during cell-cell contact formation of C2C12 myoblasts. AJ1 represents a cell-cell contact in formation (panels a-c) and AJ2 corresponds to an established cell-cell contact (panels d-f). Merged images are shown in panels c, c', f and f'. Higher magnifications of the cell-cell contact sites are shown in panels a'-f'. Bar : 10 µm.

B) C2C12 cells were left untreated (a, g) or treated with 2 mM EGTA for 60 min (b, h). The EGTA-containing medium was then replaced with calcium containing medium and cells were fixed 10 min (c, i), 30 min (d, j), 60 min (e, k) and 120 min (f, l) thereafter. Cells were stained for N-cadherin (a-f) and F-actin (g-l). Bar : 10 µm.

Figure 2 : Effects of Rac1 on N-cadherin-dependent cell-cell contact formation

A) C2C12 myoblasts transfected either with empty pEGFP (a), GFP-Rac1V12 (d) or GFP-Rac1N17 (g) were stained for N-cadherin (b, e and h) and F-actin (c, f and i). Bar : 10 µm. Panels e' and f' show enlarged views of the cell-cell contact site of panels e and f.

B) GFP-Rac1V12-transfected C2C12 myoblasts (a-f) were treated or not with 2mM EGTA 24 hours post-transfection. The EGTA-containing medium was then replaced with calcium containing medium and cells were fixed at the indicated times. Cells were stained for N-cadherin (g-l) and F-actin (m-r). Bar : 10 µm.

C) C2C12 myoblasts transfected with GFP-Rac1N17 (a-f) were processed as in B and then stained for N-cadherin (g-l) and F-actin (m-r). Bar : 10 µm.

Figure 3 : Effects of RhoA on N-cadherin-dependent cell-cell contact formation

A) C2C12 myoblasts transfected with GFP-RhoAV14 (a) or GFP-RhoAN19 (d) were stained for N-cadherin (b and e) and F-actin (c and f). Bar : 10 µm. Panels b', c', e' and f' show enlarged views of the cell-cell contact sites of the selected areas.

B) GFP-RhoAV14-transfected C2C12 myoblasts (a-f) were treated or not with 2mM EGTA 24 hours post-transfection. The EGTA-containing medium was then replaced with calcium containing medium and cells were fixed at the indicated times. Cells were stained for N-cadherin (g-l) and F-actin (m-r). Bar : 10 µm.

C) C2C12 myoblasts transfected with GFP-RhoAN19 (a-f) were stained for N-cadherin (g-l) and F-actin (m-r). Cells were either untreated (a, g and m), treated with EGTA (b, h and n) or treated with EGTA and allowed to recover in Ca^{2+}-containing medium as indicated (c-f, i-l and o-r). Bar : 10 µm.

Figure 4 : Effects of Rac1 and RhoA on the mobile pool and on the lateral mobility of N-cadherin at the cell-cell contact sites

A) Transfected C2C12 myoblasts were cell surface biotinylated at 4°C. The histrogram represents the mean level of biotinylated N-cadherin normalized to the amount of total protein from three independent experiments.

B) Shown are the first 60 seconds of photobleach recovery data on N-cadherin/GFP in AJ1 or AJ2 contacts or in cell co-expressing RFP-Rac1V12 or RFP-RhoAL63.

C) Diffusion coefficient (D) and mobile fraction (M) of N-cadherin/GFP in membrane involved in cell-cell contacts. RFP-Rac1V12 or RFP-RhoAL63-expressing C2C12 myoblasts have established cell-cell contacts for 24-36 hours. A minimum of 15 cells were analysed for each condition. Significance values (by paired Student's t test) are shown on the graph.

D) Caracteristic images of a FRAP experiment obtained in RFP-Rac1V12 or RFP-RhoAL63-expressing C2C12 myoblasts. Images before, immediatly after (asterisk), or at the indicated times after bleaching of N-cadherin/GFP are shown. The circle (1µm-diameter) on the prebleach image indicates the bleached region.

Figure 5 : Effect of inhibitors of ROCK, MLCK and Myosin 2 on N-cadherin association at cell-cell contacts

A) C2C12 myoblasts either untreated (a), or treated with the ROCK inhibitor Y27632 (b), the MLCK inhibitor ML-7 (c), the inhibitor of the Myosin 2 ATPase activity blebbistatin (d), were stained for N-cadherin. Bar : 10 µm.

B) C2C12 myoblasts, either untreated, or treated either with blebbistatin, ML-7 or Y27632, were cell surface biotinylated at 4°C. The histogram represents the mean level of biotinylated N-cadherin normalized to the amount of total protein from three independent experiments.

C) Diffusion coefficient (D) and mobile fraction (M) of N-cadherin/GFP in membrane involved in cell-cell contacts. Cells with established cell-cell contacts (AJ2) were left untreated or treated either with blebbistatin, ML-7, or Y27632. A minimum of 20 cells were analysed for each condition. Significance values (by paired Student's t test) are shown on the graph.

Figure 6 : Effect of RhoA on N-cadherin accumulation at cell-cell contacts in RD cells

A) RD cells transfected with empty pEGFP (panel a) or GFP-RhoAV14 (panel b) were stained for N-cadherin (b and d). Bar : 10 µm.

B) Graphic representation of the signal across the width of the cells (line) shown in b and d. Fluorescence intensity was analysed along the line by line scan (MetaMorph software). Positions 1 and 2 indicate the cell-cell contacts.

C) Percentage of cells which show N-cadherin localization at the sites of cell-cell contact 24 hours after transfection. Data are means +/- s.e.m. of five independent experiments (50 cells were analyzed in each experiment) with 95% confidence limits determined by Kruskall-Wallis non parametric test. RhoAV14 expression has a significant effect with $p<0.05$.

Table 1 : Effect of different treatments on N-cadherin association at the cell-cell contact sites

AJ1 : : cell-cell contact in formation often seen in subconfluent cells.

AJ2 : established cell-cell contact observed in fully contacting cells.

During the establishment of cell-cell contacts a transition from AJ1 to AJ2 is observed, which is also observed upon induction of calcium-dependent intercellular adhesion achieved as described in the first line.

Several drugs (Y27632, BDM, ML7 and blebbistatin), constitutively active Rac1 (Rac1V12), dominant negative RhoA (RhoAN19) or inhibition of N-cadherin-dependent cell-cell contacts using antibody against the extracellular domain of N-cadherin favours the formation of AJ1-type cell-cell contacts and the inhibition of the AJ1 to AJ2 transition.

Expression of constitutively active RhoA (RhoAV14) or dominant negative Rac1 (Rac1N17) impairs the appearance of the AJ1 stage and delays the AJ2 stage. RhoAV14 increases N-cadherin accumulation at the cell-cell contacts.

Material and methods

Cell culture, treatments and transfections

C2C12 myoblasts and RD rhabdomyoblasts were cultured as described (Charrasse *et al.*, 2002; Charrasse *et al.*, 2004). Cells were treated with Y27632632 (20µM, Sigma), BDM (20mM, Sigma), ML7 (3µM, Calbiochem), Blebbistatin (5µM, Calbiochem), Jasplakinolid (50nM, Calbiochem) and Cytochalasin B (CY, 2µM for 1 hour, Sigma). EGTA was used at 2mM.

Cells were plated on 18-mm-diameter glass coverslips 16-24 hours before transfection. Cells were transfected with the GFP-Rac1V12, GFP-Rac1N17, GFP-RhoAV14, GFP-RhoAN19, RFP-Rac1V12, RFP-RhoAL63 plasmids or with the pEGFPC1 empty vector (Gauthier-Rouviere *et al.*, 1998; Alberts *et al.*, 2006).

Immunocytochemistry

Cells were processed as described (Causeret et al., 2005). Images were captured with a MicroMax 1300 CCD camera (RS-Princeton Instruments, Treuton, NJ, USA) driven by the MetaMorph (v.6, Universal Imaging Corp, Westchester, PA, USA) Software. Images were processed using Adobe Photoshop and Illustrator.

Cell surface biotinylation

Cell surface proteins were biotinylated by incubating the cells with 1.5mg/ml sulfo-NHS-SS-biotin (Pierce Chemical) as described (Le et al., 1999). Cleared lysates (except 20µl corresponding to the total fraction) were incubated with streptavidin beads (Pierce Chemical) to collect biotinylated proteins. As a control streptavidin beads were incubated with non-

biotinylated cell lysates. The samples were then analysed by immunoblotting for N-cadherin. Scanned autoradiographs were quantified using Aïda/2D densitometry software.

Fluorescence recovery after photobleaching

Lateral diffusion coefficients (D) and mobile fractions (M) were measured by FRAP using a Zeiss LSM Meta 510 confocal microscope as described (Causeret et al., 2005).

References

Adams, C.L., Chen, Y.T., Smith, S.J., and Nelson, W.J. (1998) Mechanisms of epithelial cell-cell adhesion and cell compaction revealed by high-resolution tracking of E-cadherin-green fluorescent protein. J. Cell Biol. **142**, 1105-1119

Alberts, P., Rudge, R., Irinopoulou, T., Danglot, L., Gauthier-Rouviere, C., and Galli, T. (2006) Cdc42 and actin control polarized expression of TI-VAMP vesicles to neuronal growth cones and their fusion with the plasma membrane. Mol. Biol. Cell. **17**, 1194-1203

Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J. Biol. Chem. **271**, 20246-20249

Avizienyte, E., Fincham, V.J., Brunton, V.G., and Frame, M.C. (2004) Src SH3/2 domain-mediated peripheral accumulation of Src and phospho-myosin is linked to deregulation of E-cadherin and the epithelial-mesenchymal transition. Mol. Biol. Cell. **15**, 2794-2803

Braga, V.M. (2002). Cell-cell adhesion and signalling. Curr Opin Cell Biol *14*, 546-556.

Braga, V.M., Del Maschio, A., Machesky, L., and Dejana, E. (1999) Regulation of cadherin function by Rho and Rac: modulation by junction maturation and cellular context. Mol. Biol. Cell. **10**, 9-22

Braga, V.M., Machesky, L.M., Hall, A., and Hotchin, N.A. (1997) The small GTPases Rho and Rac are required for the establishment of cadherin-dependent cell-cell contacts. J. Cell. Biol. **137**, 1421-1431

Causeret, M., Taulet, N., Comunale, F., Favard, C., and Gauthier-Rouviere, C. (2005) N-Cadherin Association with Lipid Rafts Regulates its Dynamic Assembly at Cell-Cell Junctions in C2C12 Myoblasts. Mol. Biol. Cell. **16**, 2168-2180

Charrasse, S., Causeret, M., Comunale, F., Bonet-Kerrache, A., and Gauthier-Rouviere, C. (2003) Rho GTPases and cadherin-based cell adhesion in skeletal muscle development. J. Muscle Res. Cell Motil. **24**, 309-313

Charrasse, S., Comunale, F., Gilbert, E., Delattre, O., and Gauthier-Rouviere, C. (2004) Variation in cadherins and catenins expression is linked to both proliferation and transformation of Rhabdomyosarcoma. Oncogene **23**, 2420-2430

Charrasse, S., Comunale, F., Grumbach, Y., Poulat, F., Blangy, A., and Gauthier-Rouviere, C. (2006) RhoA GTPase regulates M-cadherin activity and myoblast fusion. Mol. Biol. Cell. **17**, 749-759

Charrasse, S., Meriane, M., Comunale, F., Blangy, A., and Gauthier-Rouviere, C. (2002) N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. J. Cell. Biol. **158**, 953-965

Chu, Y.S., Thomas, W.A., Eder, O., Pincet, F., Perez, E., Thiery, J.P., and Dufour, S. (2004) Force measurements in E-cadherin-mediated cell doublets reveal rapid adhesion strengthened by actin cytoskeleton remodeling through Rac and Cdc42. J. Cell. Biol. **167**, 1183-1194

Cinnamon, Y., Ben-Yair, R., and Kalcheim, C. (2006) Differential effects of N-cadherin-mediated adhesion on the development of myotomal waves. Development **133**, 1101-1112

Duband, J.L., Dufour, S., Hatta, K., Takeichi, M., Edelman, G.M., and Thiery, J.P. (1987) Adhesion molecules during somitogenesis in the avian embryo. J. Cell. Biol. **104**, 1361-1374

Ehrlich, J., Hansen, M.D.H., and Nelson, W.J. (2002) Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. Developmental Cell **3**, 259-270

Fukata, M., and Kaibuchi, K. (2001) Rho-family GTPases in cadherin-mediated cell-cell adhesion. Nat Rev Mol. Cell. Biol. **2**, 887-897

Gauthier-Rouviere, C., Vignal, E., Meriane, M., Roux, P., Montcourier, P., and Fort, P. (1998) RhoG GTPase controls a pathway that independently activates Rac1 and Cdc42Hs. Mol. Biol. Cell. **9**, 1379-1394

George-Weinstein, M., Gerhart, J., Blitz, J., Simak, E., and Knudsen, K.A. (1997) N-cadherin promotes the commitment and differentiation of skeletal muscle precursor cells. Dev. Biol. **185**, 14-24

Goichberg, P., and Geiger, B. (1998) Direct involvement of N-cadherin-mediated signaling in muscle differentiation. Mol. Biol. Cell. **9**, 3119-3131

Goichberg, P., Shtutman, M., Ben-Ze'ev, A., and Geiger, B. (2001) Recruitment of beta-catenin to cadherin-mediated intercellular adhesions is involved in myogenic induction. J. Cell Sci. **114**, 1309-1319

Hatta, K., Takagi, S., Fujisawa, H., and Takeichi, M. (1987) Spatial and temporal expression pattern of N-cadherin cell adhesion molecules correlated with morphogenetic processes of chicken embryos. Dev. Biol. **120**, 215-227

Jou, T.S., and Nelson, W.J. (1998) Effects of regulated expression of mutant RhoA and Rac1 small GTPases on the development of epithelial (MDCK) cell polarity. J. Cell. Biol. **142**, 85-100

Kimura, K., Ito, M., Amano, M., Chihara, K., Fukata, Y., Nakafuku, M., Yamamori, B., Feng, J., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) Regulation of myosin phosphatase by Rho and Rho-associated kinase (Rho-kinase). Science **273**, 245-248

Knudsen, K.A. (1990). Cell adhesion molecules in myogenesis. Curr Opin Cell Biol 2, 902-906.

Kuroda, S., Fukata, M., Fujii, K., Nakamura, T., Izawa, I., and Kaibuchi, K. (1997) Regulation of cell-cell adhesion of MDCK cells by Cdc42 and Rac1 small GTPases. Biochem. Biophys. Res. Commun. **240**, 430-435

Kusumi, A., Sako, Y., and Yamamoto, M. (1993) Confined lateral diffusion of membrane receptors as studied by single particle tracking (nanovid microscopy). Effects of calcium-induced differentiation in cultured epithelial cells. Biophys. J. **65**, 2021-2040

Lambert, M., Choquet, D., and Mege, R.M. (2002) Dynamics of ligand-induced, Rac1-dependent anchoring of cadherins to the actin cytoskeleton. J. Cell. Biol. **157**, 469-479

Le, T.L., Yap, A.S., and Stow, J.L. (1999) Recycling of E-cadherin: a potential mechanism for regulating cadherin dynamics. J. Cell. Biol. **146**, 219-232

Linask, K.K., Ludwig, C., Han, M.D., Liu, X., Radice, G.L., and Knudsen, K.A. (1998) N-cadherin/catenin-mediated morphoregulation of somite formation. Dev. Biol. **202**, 85-102

Lozano, E., Betson, M., and Braga, V.M. (2003) Tumor progression: Small GTPases and loss of cell-cell adhesion. Bioessays **25**, 452-463

Meriane, M., Charrasse, S., Comunale, F., Mery, A., Fort, P., Roux, P., and Gauthier-Rouviere, C. (2002) Participation of small GTPases Rac1 and Cdc42Hs in myoblast transformation. Oncogene **21**, 2901-2907

Meriane, M., Roux, P., Primig, M., Fort, P., and Gauthier-Rouviere, C. (2000) Critical activities of Rac1 and Cdc42Hs in skeletal myogenesis: antagonistic effects of JNK and p38 pathways. Mol. Biol. Cell. **11**, 2513-2528

Merlino, G., and Helman, L.J. (1999) Rhabdomyosarcoma--working out the pathways. Oncogene **18**, 5340-5348

Ridley, A.J., Paterson, H.F., Johnston, C.L., Diekmann, D., and Hall, A. (1992) The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell **70**, 401-410

Sahai, E., and Marshall, C.J. (2002) ROCK and Dia have opposing effects on adherens junctions downstream of Rho. Nat. Cell. Biol. **4**, 408-415

Sako, Y., Nagafuchi, A., Tsukita, S., Takeichi, M., and Kusumi, A. (1998) Cytoplasmic regulation of the movement of E-cadherin on the free cell surface as studied by optical tweezers and single particle tracking: corralling and tethering by the membrane skeleton. J. Cell. Biol. **140**, 1227-1240

Schwartz, M. (2004) Rho signalling at a glance. J. Cell Sci. **117**, 5457-5458

Shewan, A.M., Maddugoda, M., Kraemer, A., Stehbens, S.J., Verma, S., Kovacs, E.M., and Yap, A.S. (2005) Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. Mol. Biol. Cell. **16**, 4531-4542

Takaishi, K., Sasaki, T., Kotani, H., Nishioka, H., and Takai, Y. (1997) Regulation of cell-cell adhesion by rac and rho small G proteins in MDCK cells. J. Cell. Biol. **139**, 1047-1059

Vasioukhin, V., Bauer, C., Yin, M., and Fuchs, E. (2000) Directed actin polymerization is the driving force for epithelial cell-cell adhesion. Cell **100**, 209-219

Vasioukhin, V., and Fuchs, E. (2001) Actin dynamics and cell-cell adhesion in epithelia. Curr. Opin. Cell. Biol. **13**, 76-84

Volberg, T., Geiger, B., Kartenbeck, J., and Franke, W.W. (1986) Changes in membrane-microfilament interaction in intercellular adherens junctions upon removal of extracellular Ca2+ ions. J. Cell. Biol. **102**, 1832-1842

Yap, A.S., and Kovacs, E.M. (2003) Direct cadherin-activated cell signaling: a view from the plasma membrane. J. Cell. Biol. **160**, 11-16

Zhang, J., Betson, M., Erasmus, J., Zeikos, K., Bailly, M., Cramer, L.P., and Braga, V.M. (2005) Actin at cell-cell junctions is composed of two dynamic and functional populations. J. Cell Sci. **118**, 5549-5562

Zhong, C., Kinch, M.S., and Burridge, K. (1997) Rho-stimulated contractility contributes to the fibroblastic phenotype of Ras-transformed epithelial cells. Mol. Biol. Cell. **8**, 2329-2344

Abbreviations used :

AJ1, cell-cell contact in formation

AJ2, established cell-cell contact

Ca^{2+}, calcium ion

D, diffusion coefficient

FRAP, fluorescent recovey after photobleaching

GFP, green fluorescence protein

M, mobile fraction

MLCK, myosin light chain kinase

RMS, rhabdomyosarcoma

ROCK, Rho-associated kinase