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Identification of a bipartite focal adhesion localization signal in RhoU/Wrch-1, a Rho family GTPase that regulates cell adhesions and migration

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Background information

Rho GTPases are important regulators of cytoskeleton dynamics and cell adhesion.

RhoU/Wrch-1 is a Rho GTPase, which shares sequence similarities with Rac1 and Cdc42 but has also extended amino- and carboxy-terminal domains. Whereas the N-terminal extension promotes binding to SH3 domain-containing adaptors, the C-terminal extension mediates membrane targeting through palmitoylation of its non-conventional CAAX-box. RhoU/Wrch-1 possesses transforming activity, which is negatively regulated by its N-terminal extension and depends on palmitoylation.

Results

In this study, we show that RhoU is localized to podosomes in osteoclasts and c-Src expressing cells, and to focal adhesions of HeLa cells and fibroblasts. Whereas the N-terminal extension and the palmitoylation site were dispensable, the C-terminal extension and effector binding loop were critical for RhoU targeting to focal adhesions. Moreover, the number of focal adhesions was reduced and their distribution changed upon expression of activated RhoU. Conversely, RhoU silencing increased the number of focal adhesions. As RhoU was only transiently associated with adhesion structures, this suggests that RhoU may modify adhesion turnover and cell migration rate. Indeed, we found that migration distances were increased in cells expressing activated RhoU and decreased when RhoU was knocked-down.

Conclusions

Our data indicate that RhoU localizes to adhesion structures, regulates their number and distribution and increases cell motility. It also suggests that the RhoU effector binding and C-terminal domains are critical for these functions.

INTRODUCTION

Fundamental biological processes such as development, chemotaxis or wound healing require that cells move around in a highly coordinated and regulated manner. A key step in cell migration is the regulation of adhesion structures that need to be assembled at the leading edge and disassembled at the rear to allow effective cell movement. As cells migrate, they form new adhesion points that anchor the cell to the substrate through integrin clustering, which, in turn, initiates assembly of multi-molecular complexes and link the extracellular matrix to the actin cytoskeleton. Focal complexes are small integrin clusters that are formed first at the cell contact with extracellular matrix. They may disappear or evolve into larger structures, the focal adhesions (FAs), from which contractile forces will be generated to allow cell body translocation (Geiger et al., 2001). Spatially restricted cues may account for the differential regulation of adhesion structures in a single cell. Rho GTPases are key regulators and integrators of this process (Pertz, Hahn, 2004). They act as molecular switches that are positively regulated by guanine nucleotide exchange factors (GEFs), which catalyze the exchange of GDP for GTP, and are negatively regulated by GTPases activating proteins (GAPs), which increase their intrinsic GTPase activity. As a consequence of a conformational change upon GTP binding, conserved residues in the effector loop are exposed and mediate binding to GTPase effectors (Feltham et al., 1997; Ihara et al., 1998). Once bound to GTP, they can integrate signals emanating from different stimuli (Bishop and Hall, 2000).

Regulators should also be differentially expressed to allow specific dynamics of actin and adhesion structures in migrating cells. Spatio-temporal studies of Cdc42, Rac1 and RhoA activities have, indeed, established a correlation between the site of activation and the specific function of Rho GTPases in actin cytoskeleton organization. For example, Cdc42 has been implicated in filopodia formation (Nobes and Hall, 1995) and optimal levels of activation are

found at the base of filopodia during cell adhesion (Itoh et al., 2002, Nalbant et al., 2004). Rac1 promotes lamellipodia formation at the leading edge of migrating cells (Nobes and Hall, 1999) and its activation occurs primarily there (Kraynov et al., 2000; Gardiner et al., 2002; del Pozo et al., 2002; Kurokawa et al., 2004). Finally, RhoA increases cell contractility (Chrzanowska-Wodnika and Burridge, 1996) and its activation occurs where contractile forces are required, i.e. at the cell periphery where cortical actin tensions are present and at the rear of the migrating cell to allow cell retraction (Pertz et al., 2006). In addition, Rho GTPases have been localized to adhesion structures, either podosomes or FAs (Linder et al., 1999; Berdeaux et al., 2004; ten Klooster et al., 2006; Chang et al., 2007). Interestingly, the recruitment of Rac1 into FAs requires a proline stretch in its hypervariable C-terminal region, which binds to the SH3 domain of β PIX (ten Klooster et al., 2006; Chang et al., 2007). Thus, the hypervariable region of Rho GTPases mediates specific subcellular targeting by both differential lipid modifications (Michaelson et al., 2001; Heo et al., 2006) and recruitment of specific signaling complexes that may be independent from lipid modifications (ten Klooster et al., 2006).

RhoU/Wrch-1 has been recently identified as a new Rho GTPase member (Tao et al., 2001). Although RhoU/Wrch-1 shares sequence similarity with Rac1 and Cdc42, it possesses N- and C-terminal extensions that confer specific properties and distinguish it from Rac1 and Cdc42. Indeed, its N-terminal extension, which regulates RhoU transforming activity, contains PxxP motifs and is involved in the binding of adaptor proteins such as Nck1 or Grb2 (Saras et al., 2004; Shutes et al., 2004). In addition, RhoU has an unusual CAAX box, which, in contrast to Rac1 and Cdc42, is palmitoylated instead of prenylated (Berzat et al., 2005). The lack of palmitoylation prevents RhoU association with cell membranes, alters its transforming activity and its ability to activate PAK1 (Berzat et al., 2005). Like Cdc42, RhoU induces actin reorganization and formation of filopodia (Tao et al., 2001). However, RhoU

may recruit different effectors since, in contrast to Cdc42, it does not bind to the isolated CRIB domain of WASP *in vitro* (Aspenström et al., 2004). This suggests that, despite similar biological functions, RhoU may activate a different set of effectors.

We recently showed that RhoU mRNA is induced during the differentiation of macrophages into osteoclasts (Brazier et al., 2006). To gain insight into the function of RhoU, we first sought to characterize the subcellular localization of GFP-tagged RhoU. In osteoclasts, GFP-RhoU accumulated both at the podosome belt, which corresponds to a peripheral clustering of podosomes, and all around isolated podosomes. Similarly, RhoU localized to podosomes in c-Src expressing cells. As most cell types maintained in culture have FAs rather than podosomes, we checked whether RhoU association with adhesion structures was restricted to podosomes. We found that RhoU localized to FAs, but not to focal complexes, and whereas this localization did not require an intact palmitoylation site or the N-terminal extension, an intact effector binding site and the C-terminal extension were needed. Finally, in NIH3T3 cells, activated RhoU caused dramatic loss and redistribution of FAs, both in cells maintained on their substratum or in response to adhesion. A reverse phenotype was observed when RhoU was silenced. Finally, expression of activated RhoU increased fibroblast cell motility. Altogether, our results highlight new properties for RhoU in the control of cell adhesion and migration.

RESULTS

RhoU is localized to adhesion structures in osteoclasts

We have recently shown that among the 13 small Rho GTPases expressed in osteoclasts, RhoU has a crucial function in their differentiation (Brazier et al., 2006). To better define the role of RhoU, we decided first to characterize its subcellular distribution in osteoclasts. For that purpose, we made use of retroviruses to express the green fluorescent protein (GFP) alone or fused to the N-terminus of RhoU proteins (wild type or mutants) in RAW264.7 cells. After selection and a 5-day treatment with RANKL, we obtained osteoclasts expressing GFP alone, GFP-RhoU WT, or the GFP RhoU Q107L and GFP RhoU T63N mutants, which are analogous to the active (Q61L) and the dominant negative (T17N) Cdc42 mutants, respectively. Osteoclasts seeded on glass organize their actin cytoskeleton into a podosome belt (Destaing et al., 2003). Indeed, as shown in Fig.1, immunofluorescence analysis of GFP-expressing osteoclasts revealed that F-actin accumulated at the cell periphery and was surrounded by vinculin (Fig.1, A). Despite a slight accumulation of GFP with the filamentous actin at the cell periphery, GFP was essentially excluded from the vinculin-labeled area (Fig.1, A, insets). On the other hand, GFP-RhoU WT (Fig.1B, inset) and GFP-RhoU Q107L (Fig.1C, inset a, and b) colocalized with vinculin. In isolated podosomes, GFP-RhoU Q107L and vinculin surrounded the actin dot (inset b, Fig.1C). Importantly, the GFP-RhoU T63N mutant did not colocalize with vinculin (Fig. 1D, inset). These observations indicate that RhoU is localized to adhesion structures of osteoclasts and that the T63N mutation prevents its accumulation at the podosome belt.

RhoU localizes to Src-dependent podosomes

Podosomes have now been identified in many cell types (Linder and Aepfelbacher, 2003). Rosettes of adhesion structures and podosomes have been originally described in fibroblasts transformed by the Rous Sarcoma Virus (David-Pfeuty and Singer, 1980; Tarone et al., 1985). Despite similarities between podosomes in cells from the myeloid lineage and those induced by c-Src, their half-life and size are different (Linder and Aepfelbacher, 2003). We, thus, wanted to see whether RhoU could also be associated with Src-induced podosomes. We expressed a constitutive activated form of Src (SrcY527F) in HeLa and NIH3T3 cells. Although SrcY527F induces two different types of F-actin structures, i.e. isolated podosomes in HeLa cells (Fig.2) or rosettes of podosomes in NIH3T3 cells (not shown), RhoU was found at the periphery of both structures. In HeLa cells, GFP-RhoU WT could not be clearly localized around the podosomes (not shown), whereas GFP-RhoU Q107L surrounded the actin core of isolated podosomes (Fig.2A, B), but was not associated with all podosomes. Of three clearly visible Src-positive F-actin dots (Fig.2A), GFP-RhoU Q107L completely surrounded only one, partially the second one (arrowheads inset Fig.2A) and was excluded from the third one (arrows). To further characterize the GFP-RhoU-positive podosomes, we labeled Src-transfected cells for vinculin (Fig.2B) and paxillin (not shown), two proteins known to accumulate around the actin core of podosomes. Some F-actin dots were devoid either of vinculin (Fig.2B) or paxillin (not shown) and, as shown in Fig 2B (inset), actin dots surrounded by vinculin (arrowheads) were also surrounded by GFP-RhoU Q107L (arrowheads inset Fig.2B) whereas actin dots devoid of vinculin were not. Similarly to adhesion structures in osteoclasts, GFP-RhoU T63N was never observed in podosomes (not shown). These results indicate that RhoU can associate both with adhesion structures of osteoclasts and with Src-induced podosomes.

RhoU is targeted to focal adhesions

In contrast to myeloid cells or Src-transformed cells, most cell types develop focal adhesions (FAs) to adhere and migrate in culture. To determine whether the association of RhoU with adhesion structures was podosome-specific, we expressed GFP-RhoU in HeLa cells, which are rich in FAs. As previously reported (Shutes et al., 2004), GFP-RhoU (WT, Q107L, T63N) was associated with vesicular compartments that partially colocalize with transferrin-positive vesicles in internalization experiments (not shown). Moreover, in cells with low to moderate levels of exogenous proteins, in addition to the vesicular localization, GFP-RhoU WT or Q107L were found in a peripheral streak-like pattern at the substratum, an arrangement reminiscent of FAs (Fig.3). A similar pattern was observed also with myc-tagged RhoU (not shown). To confirm RhoU localization in FAs, we labeled cells for paxillin (Fig.3) or vinculin (not shown). GFP-RhoU WT (Fig.3A) and GFP-RhoU Q107L (Fig.3B) colocalized with paxillin whereas GFP-RhoU T63N was never found in FAs but only in the vesicular compartment (Fig.3C). Fig.3D and 3E show RhoUQ107L in FAs at the basal plane (Fig.3D) and in vesicles 2 μm above the substratum (Fig.3E). In around 50% of transfected cells, RhoU was localized to FAs (Fig.3F) and the number of cells in which RhoU was associated with FAs, did not vary significantly between GFP-RhoU WT and GFP-RhoU Q107L expressing cells. These observations indicate that RhoU is able to associate with both types of adhesion structures: podosomes and focal adhesion plaques.

The C-terminal extension is required for RhoU targeting to focal adhesions

Rac1 also localizes to FAs (ten Klooster et al., 2006 and supplementary Fig.S1) and a stretch of 3 prolines at its C-terminus end could be important for this localization (ten Klooster et al., 2006). RhoU lacks these 3 prolines, but has additional motifs that may be involved in its recruitment to adhesion structures (see supplementary Fig.S1, A). In particular, the N-terminal extension PxxP motifs are potential SH3-binding motifs (Saras et al., 2004; Shutes et al., 2004). To assess whether the N- and C-terminal extensions of RhoU are required for its FA localization, we generated mutants in which the first 47 amino acids were deleted (Δ N47_RhoU Q107L), alone or together with the last 35 amino acids (Δ N47_RhoU Q107L_ Δ C223; Fig.4A). In addition, as membrane localization of Rho GTPases is critical for their function, we substituted cysteine 255 and 256 for serines (Δ N_RhoU Q107L C255,256S) to prevent their palmitoylation, a lipid modification which is crucial for RhoU membrane association (Berzat et al., 2005). The distribution of RhoU Q107L truncated mutants was compared to that of paxillin (Fig.4). As shown in Fig.4B, Δ N_RhoU Q107L is localized to FAs, indicating that, in contrast to Rac1, SH3 domain-containing adaptors are dispensable for targeting of RhoU to FAs. Regardless of the presence (not shown) or the absence of the N-terminal extension, RhoU deleted of the C-terminal extension was never found in FAs. Nonetheless, fusion of RhoU C-terminal domain to GFP was not sufficient to localize GFP to FAs. As expected, this protein accumulated in large vesicles above the substratum (Fig.4E). In addition, the C255,256S mutation did not prevent RhoU localization to FAs, although, as described (Shutes et al., 2004), it showed a diffuse cytoplasmic localization and no association with vesicles (Fig.4D). Finally, the proportion of transfected cells in which RhoU mutants associated with FAs was similar to that of the full length protein (Fig.4H). These data

indicate that the palmitoylation is not necessary for RhoU targeting to FAs and that the C-terminal extension is required but not sufficient.

Effector binding is required for RhoU targeting to focal adhesions

As a single mutation (T63N) in RhoU was sufficient to abolish accumulation of RhoU at FAs (Fig.3) and prevent its binding to PAK1 (Saras et al., 2004, Fig.4I), we hypothesized that, together with the C-terminal extension, binding to effectors may be crucial to localize RhoU to FAs. To further address this hypothesis, we generated RhoU effector loop mutants (T81S, F83A, F86C, equivalent to T35S, F37A, Y40C in Rac1) and evaluated their ability to bind to the CRIB domain of PAK in vitro by GST-CRIB pull-down assay. As expected, the combination of 3 mutations in the effector loop (3M mutant) abolished binding to the CRIB domain. In contrast to Rac1 (Lamarche et al., 1996; Westwick et al., 1997), all single mutants were still able to bind to the CRIB domain (Fig.4I). Moreover, all single mutants localized to FAs (Fig.4H), whereas the 3M mutant did not in 85% of transfected cells (Fig.4H). When associated with FAs, the 3M mutant showed a highly reduced fluorescence intensity compared to that of GFP-RhoU Q107L (Fig.4F). Therefore, there is a strong correlation between the capacity of RhoU to bind to effectors and its localization to FAs. As Cdc42 is not found in FAs, but both RhoU and Cdc42 bind to PAK1, we asked whether the C-terminal extension of RhoU was sufficient to drive Cdc42 into FAs. We, thus, fused the C-terminal extension of RhoU to Cdc42 and analyzed the subcellular localization of the chimera. Despite its ability to bind to the CRIB domain in vitro (Fig.4I), the chimera localized to small vesicles throughout the cell and was never found associated with FAs (Fig.4G). Similarly, although unable to localize to FAs, C-terminal deletion mutants were still able to bind to the CRIB

domain (Fig.4I). Altogether these data show that the effector loop and the C-terminal domain of RhoU are necessary but not sufficient to target RhoU to FAs.

RhoU reduces the number of adhesion structures in cells

To address whether RhoU affects FA formation, we expressed GFP alone or fused to RhoU WT, Q107L or T63N in NIH3T3 cells. 36 hours later, cells were fixed and labeled for F-actin and paxillin at steady state. GFP-RhoU WT (not shown) or Q107L-expressing cells (Fig.5A) showed a reduction in the number of paxillin-positive FAs compared to GFP expressing cells. The central adhesion structures were absent or highly reduced. We validated these observations by semi-automatic morphometric analysis of adhesion structures in 30 to 40 cells expressing the recombinant proteins (see Material and Methods). Fig.5B shows that expression of GFP-RhoU Q107L or, to a lesser extent, of GFP-RhoU WT significantly reduced the total number of adhesion structures compared to GFP alone. The distribution of FAs was also affected since the number of central adhesion sites was reduced in GFP-RhoU Q107L expressing cells (Fig.5C). The size of adhesion structures and the area of GFP-RhoU Q107L expressing cells were however unchanged (not shown). These data indicate that RhoU regulates the number of adhesion structures at the steady state. To determine which step of adhesion formation RhoU may be involved in, we performed adhesion assays on vitronectin. GFP-RhoU expressing cells were detached from the culture dish, maintained 1 hour in suspension and seeded back onto vitronectin-coated coverslips. Cells were fixed after 15, 30 and 60 minutes and labeled for paxillin to follow adhesion structure formation. At 15 and 30 minutes, GFP-RhoU Q107L (Fig.6A) was associated with adhesions localized more towards the center of the cell (arrowheads inset), but was excluded from paxillin-containing adhesions at the extreme periphery of the cell (arrows inset). At 60 minutes most adhesions contained

both paxillin and RhoU (arrows inset), but the number of paxillin-containing adhesion sites was reduced. We confirmed this observation by morphometric analysis and found that the number of adhesion structures was significantly reduced in cells expressing GFP-RhoU WT or GFP-RhoU Q107L compared to GFP or GFP-RhoU T63N (Fig.6B). The distribution of adhesion structures was also significantly affected with a loss of central adhesions in GFP-RhoU Q107L (Fig.6C). We next knocked-down RhoU expression with retroviruses expressing a specific RhoU shRNA (shRhoU, Brazier et al., 2006). In the absence of a specific anti-RhoU antibody, we monitored the efficiency of RhoU silencing after cell selection by real-time PCR amplification (Brazier et al., 2006), which showed 88% reduction in comparison to control cells expressing Luciferase shRNA(shLuc, Fig.6D). shRNA expressing cells were prepared for adhesion assays on vitronectin. Adhesion structure formation was then monitored on fixed cells by paxillin labeling. At 30 minutes, we observed that adhesion structures were more frequent and their distribution more central in cells depleted for endogenous RhoU (Fig.6E). At 60 min, no significant differences were found either in the number of adhesion structures or in their distribution (Fig.6F, G). These data indicate that RhoU regulates the number and distribution of paxillin-containing adhesion sites.

RhoU increases cell motility

RhoU effects on the number and distribution of adhesion structures suggest that it might also influence cell migration. To test this hypothesis, we used the NIH3T3 cells that stably express GFP alone or GFP-RhoU proteins. Cells were seeded at low density on vitronectin-coated wells and random cell movement was recorded under phase contrast microscope for 17,5 hours (Fig.7A,B) or 20,5 hours (Fig.7C). Fig.7A shows a frame taken off the recorded sequence (left) and tracks of individual cells (right). GFP-RhoU Q107L-expressing cells

showed a bipolar morphology (Fig.7A) and faster migrating rate compared to GFP-expressing cells (Fig.7B). As for the number of FAs, whereas GFP-RhoU WT-expressing cells had an intermediate migration rate compared to GFP or GFP-RhoU Q107L, GFP-RhoU T63N-expressing cells did not show any significant change in migration rate compared to GFP (Fig.7B). Finally, to assess the contribution of endogenous RhoU, migration assays were performed using shRhoU expressing cells. We found that RhoU silencing slightly but significantly decreased the migration rate compared to control cells (Fig. 8C). Altogether, our data indicates that RhoU targeting to FAs affects cell migration.

Online Supplementary material

The stretch of prolines found at the C-terminus of Rac1 is absent from RhoU and reduced to two residues in Cdc42 (Supplemental Fig.S1A). To compare the localizations of Cdc42, Rac1 and RhoU in our conditions, we transfected HeLa cells with constructs encoding activated Cdc42, Rac1 and RhoU mutants fused to the GFP (GFP-Cdc42Q61L (Fig.S1B), GFP-Rac1Q61L (Fig.S1C) and GFP-RhoU Q107L (Fig.S1D)). Immunofluorescence analysis following paxillin staining showed that RhoU and Rac1 each colocalized with paxillin in adhesion structures (Fig. S1C, D). In contrast, as already described (Erickson, 1996; Nalbant et al., 2004), Cdc42 was at the plasma membrane, filopodia (arrows) and vesicular compartments but was excluded from paxillin-positive FAs (Fig. S1B). Thus, in our conditions, Rac1 and RhoU but not Cdc42 are found in adhesion structures.

DISCUSSION

In this study, we show that, in addition to its endomembrane localization, RhoU is also recruited to adhesion structures, either podosomes or FAs. The N-terminal extension and the palmitoylation motif are not necessary for targeting RhoU to FAs, whereas the C-terminal extension and effector binding domain are required but not sufficient. Moreover, over-expression of RhoU reduces the number of FAs and redistributes them at the cell periphery in cells maintained on their substratum or in response to adhesion stimuli. We also established that RhoU is excluded from focal complexes and is recruited later into FAs. Finally, we correlated the localization and expression of RhoU with an increase in cell migration.

Does RhoU alter cell adhesion dynamics?

We found that RhoU is localized around the actin core of podosomes together with vinculin and paxillin in osteoclasts. In c-Src expressing cells, RhoU was never found around the actin dots that resemble the actin core of podosomes and are devoid of paxillin and vinculin. This observation suggests that the association of RhoU with podosomes may be transient. Early steps in podosome biogenesis require the recruitment of actin-binding proteins and proteins involved in actin nucleation and polymerization such as Arp2/3, or WASP. Proteins like vinculin or zyxin are only transiently associated with podosomes. They are absent from early events of podosome assembly and removed before podosome disassembly (Kaverina et al., 2003; Burgstaller and Gimona, 2004). RhoU temporal and spatial localization suggests that, like vinculin or zyxin, it may be excluded from early and late events responsible for podosome assembly or disassembly. Similarly, FA biogenesis in migrating cells or in response to adhesion relies on sequential recruitment of signaling complexes that promote

first the assembly of small focal complexes (Zamir et al., 2000; Rottner et al., 2001). We found that RhoU was excluded from focal complexes during the early stage of the adhesion assay and recruited later in FAs. As we used GFP-tagged proteins, we cannot formally exclude that, earlier on, RhoU was not detected due to insufficient accumulation of proteins. Nonetheless, our findings point to an enrichment of RhoU at specific stages of the adhesion process and we propose that RhoU association is transient. In addition, RhoU may increase adhesions turnover since expression of RhoU Q107L and, to a lesser extent, of RhoU WT reduced the number of adhesion structures in cells either at steady state or in response to adhesion stimuli. In contrast, RhoU silencing increased the number of adhesion structures at early time points of the adhesion assay. Recent work demonstrated that FA total number is related to extracellular matrix density and to its life span (Gupton and Watermann-Storer, 2006), therefore, at identical extracellular matrix concentrations, changes in the number of FAs should reflect changes in their dynamics. The implication of RhoU in their turnover is further suggested by the redistribution of adhesion structures towards the cell periphery when RhoU is expressed, and the opposite when RhoU is silenced. As the most mature FAs are mainly localized away from the cell periphery (Zamir et al., 2000), RhoU may either prevent FA maturation resulting in faster disassembly or directly promote their disassembly. Finally, increase in FA turnover is also consistent with the increase in cell migration that we observe in RhoU expressing cells. While submitting our study, Chuang et al., (2007) reported identical results in HeLa cells maintained at steady state. Combined with our data, these new study indicate that RhoU may regulate cell migration through increase in adhesion turnover.

Does RhoU require a specific effector for its localization and function?

We have shown that the C-terminal extension and the effector loop cooperate in targeting RhoU to FAs. Impairing the binding to the CRIB domain of PAK prevented RhoU to localize to FAs., However, the C-terminal deletion mutant of RhoU, which was still capable of binding, did not localize to FAs, indicating that both were required for targeting RhoU to FAs. The inability to localize to FAs of our Cdc42-RhoU chimera, in which Cdc42 C-terminus was replaced with that of RhoU and which still binds to the CRIB domain, further confirmed that the PAK1 binding domain and the C-terminus of RhoU are both necessary but not sufficient to target RhoU to FAs. Since RhoU and PAK1 share several functional similarities, it is tempting to speculate that PAK1 is the primary effector to mediate RhoU effects. Indeed, PAK1 can localize to FAs, promote their disassembly (Manser et al., 1997) and increase cell migration (Kiosses et al., 1999). Chuang et al. (2007) also reported that PAK1 and PAK2 silencing resulted in a FAs phenotype similar to the one observed after RhoU depletion. However, whether RhoU mediates its effect on FAs through PAK activation remains to be proved and we can not rule out the requirement of a specific effector. Indeed, whereas most residues that drive the specificity of the interaction between Rho GTPases and their effector are located at the interface between the effector loop and the CRIB domain (Mott et al., 1999; Abdul-Manan et al., 1999; Morreale et al, 2000), residues in the hypervariable region may also be critical (Li et al., 1999; Owen et al., 2000; Elliot-Smith et al., 2005). If we take into consideration the divergence of RhoU hypervariable region, its inability, unlike Cdc42, to bind to the CRIB domain of WASP (Aspenström et al., 2004 and our unpublished results), and the failure of the Cdc42-RhoU chimera to localize to FAs, it is reasonable to think that RhoU might not share the same set of effectors as Rac1 and Cdc42 and might need a specific one to mediate its action on FAs and cell migration. Moreover, in their GTP-bound form, Rho

GTPases can also bind to Rho GAP regulators, which are required for their inactivation. Although RhoU may be constitutively bound to GTP, it is nonetheless further activated by serum stimulation (Shutes et al., 2004). This may highlight a change in RhoGAP-stimulated GTPase activity. As several Rho GAPs localize to FAs (Kawai et al., 2004; Lavelin and Geiger, 2005; LaLonde et al., 2006) and are potentially involved in cell migration (LaLonde et al., 2006), it would be interesting to know whether RhoGAPs control RhoU activity. Finally, RhoU may also act as a scaffold to activate proteins important for adhesion dynamics. This could be particularly relevant in the context of osteoclasts' adhesion structures. Indeed, WASP and its partner WIP are crucial for podosome assembly (Linder et al., 2000; Chou et al., 2006) and both interact with Nck1 (Anton et al., 1998). The fact that Grb2 can be found in podosomes (Spinardi et al., 2004) and that binding to Nck1 is sufficient for WASP activation (Tomasevic et al., 2007), it indicates that RhoU may bring adaptors in podosomes that can activate proteins important for podosome dynamics. Our future goal will be to determine by which effectors and mechanisms RhoU alters adhesion dynamics and cell motility.

MATERIALS AND METHODS

DNA constructs

RhoU WT, Q107L and T63N were isolated from pRK5-myc (a gift from P. Aspenström, Saras et al., 2004) as BamHI/PstI fragments and subcloned in pEGFP-C1 between the BglII/PstI sites. All RhoU mutants were obtained by polymerase chain reaction amplification using pRK5-myc-RhoU Q107L as a template. Amplified fragments were subcloned in pEGFP vectors (BD Biosciences Clontech, Palo Alto). PCR-mediated mutagenesis was used to change cysteines 255 (TGC) and 256 (TGT) into serines (TCA and AGC respectively). The Cdc42 RhoU chimera was obtained by fusing amino acids 1 to 162 of Hs Cdc42 Q61L to the last 50 amino acids of Hs RhoU. The RhoU Q107L single or triple point effector loop mutants were obtained by mutating T81 (ACT), F83 (TTC) and F86 (TTC) to S (AGT) A (GCC) and C (TGC), respectively.

RhoU Q107L Δ C was obtained by deleting the 35 carboxy-terminal amino acids of human RhoU Q107L. Δ NRhoU Q107L Δ C was generated by further deletion of amino acids 1 to 47. For retroviral expression vectors, GFP and GFP fused RhoU constructs were subcloned into pMXs vectors (Kitamura et al., 2003). All constructs were verified by sequencing.

Real-time PCR analyses

DNaseI-treated total RNAs were extracted using the High Pure RNA Isolation kit (Roche Diagnostics). To generate cDNA, RNA was primed with 10-mer random primers and reverse transcription catalyzed using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR amplification was performed with an Mx3000p PCR apparatus (Stratagene) using the

Platinum Taq DNA polymerase (Invitrogen) and SYBR Green I (Bio Wittaker) as described (Brazier et al., 2006). Primer pairs used to amplify RhoU and Gapdh were described previously (Brazier et al., 2006). Real time PCR measures to quantify cDNAs were done in triplicate and the 95% confidence limits of the ratios to Gapdh were determined by Student's *t*-test.

Cell culture, infection and transfection

HeLa and NIH3T3 cells were cultured in DMEM supplemented with 2mM glutamine and 10% fetal calf serum (Hyclone). Osteoclasts were obtained as previously described (Brazier et al., 2006). Briefly, RAW 264.7 were maintained in α -MEM supplemented with 10% serum and 2mM glutamine. Cells were maintained for 5 days in presence of 25 ng/ml soluble RANKL (Peprotech) at 37°C and 5% CO₂ in a humidified incubator. For transfection experiments, HeLa cells were seeded on glass coverlips 24 hours prior to transfection with JetPEI (QBiogen). Transfection was performed accordingly to manufacturer's procedure. For a 35 mm dish, total amount of DNA was brought to 3 μ g by combining 0.1 μ g of plasmid of the protein of interest and 2.9 μ g of salmon sperm DNA as a carrier. Cells were processed for immunofluorescence or Western blot analysis 24 hours after transfection.

Retrovirus production was performed as described (Brazier et al., 2006). Briefly, the pMXs vector containing the indicated GFP constructs or the pSiren-RetroQ vector (BD Biosciences Clontech), containing short hairpin RNA targeting either the firefly Luciferase (Clontech) or the RhoU GTPase (Brazier et al., 2006), were co-transfected in 293T cell lines with the Friend MLV Gag-Pol expression vector pC57GP and the VSV-G envelop glycoprotein expression vector pCSIG. RAW 264.7 or NIH3T3 cells were infected with viral supernatant and selected for expression of the indicated constructs with 3 μ g/ml or 10 μ g/ml puromycin, respectively.

Indirect immunofluorescence and morphometry analysis

Differentiated osteoclasts were detached from plastic culture dishes using 1 mM EDTA in PBS (pH 7.4) and seeded on coverslips for 36 hours before processing. NIH3T3 or HeLa cells were fixed at room temperature with 4% formaldehyde (w/v) in PBS for 10 minutes. Cells were then permeabilized with 0.1% Triton-X-100 (v/v) in PBS for 5 minutes, washed and incubated with monoclonal anti-paxillin (BD Transduction laboratory), monoclonal anti-vinculin (Sigma) or rabbit polyclonal anti-cst1 for Src (a kind gift from S. Roche) diluted with 2% BSA(w/v) in PBS for 45 minutes. After 3 washes in PBS, primary antibodies were revealed with Alexa546-conjugated goat anti-mouse or anti rabbit antibodies. When mentioned, Alexa350-conjugated phalloidin (Invitrogen) was added to reveal F-actin. Cells were mounted in Mowiol® 40-88 (Sigma) and observed under an upright DMRA microscope (Leica microsystems) using an x100 objective (NA 1.40, Leica microsystems). Images were captured with a CCD MicroMax 1300 Y/HS camera (Roper Scientific) controlled by Metamorph 7.0 software (Princeton Scientific Instrument). For FAs morphometry analysis, between 20 and 30 fields were randomly chosen. Paxillin labeling was set up to obtain the minimal background in control conditions. Cells were examined with an x63/1.3 NA objective and individual cells present in the field were extracted for further analysis. Fluorescent images were deconvolved using the maximum likelihood estimation (MLE) algorithm (Huygens, Scientific Volume Imaging). As expression of GFP-RhoU Q107L increases cytoplasmic labeling of paxillin, using Metamorph, we normalized all pictures by flattening the background (pixel size=3) and subtracting background (value of 1000 from a 16 bits picture) for selective removal of objects that were not identified as adhesions. An

integrated morphometric analysis was performed on thresholded images to select classified objects of a size range of $5 \leq N \leq 1E08$.

The distance between all paxillin-positive adhesions and the cell periphery was determined by an Euclidean distance map (EDM) which was obtained by manually delineating the cell boundaries as determined by paxillin or GFP labeling, converting the resulting region to a binary image and inverting it to generate the EDM. Regions defined as adhesions by morphometric analysis were transferred on the EDM and the minimum intensity of each individual region corresponds to its closest distance from the cell edge.

GST-CRIB pull down and Western blot

Affinity precipitation assays were performed as previously described (Ory et al., 2000) except that the CRIB domain of the mouse PAK3 protein (amino acids 52-161) fused to the GST was used. Briefly, HeLa cells expressing RhoU constructs were rapidly washed in ice-cold PBS and lysed on ice with 50 mM Tris-HCl (pH 7.4), 2 mM MgCl₂, 1% Triton X-100, 10% glycerol, 100 mM NaCl and proteases inhibitor cocktail (Sigma). Lysates were centrifuged for 5 minutes at 17000 *g* at 4°C and aliquots were taken from the total cell lysate to determine protein input. 20 µg of bacterially produced GST-CRIB fusion protein bound to glutathione-coupled Sepharose beads were added to the cell lysate and maintained for 30 minutes at 4°C. Beads and proteins bound to the fusion protein were washed 4 times in lysis buffer, eluted in Laemmli buffer and then analyzed for bound GFP-RhoU by Western blotting using a monoclonal antibody mixture directed against GFP (Roche).

Cell adhesion assay and time lapse imaging

NIH3T3 cells expressing the indicated constructs were detached by trypsination, harvested and maintained in suspension for 1 hour in serum-free DMEM supplemented with 1% BSA at 10^4 cells/ml. Coverslips (immunofluorescence) or plastic culture dishes (time lapse imaging) were coated for 1 hour with 5 μ g/ml human vitronectin (R&D Systems) in PBS (pH 7.4), washed once in PBS and incubated for an additional hour in PBS containing 1% BSA. Cells were plated either on vitronectin-coated coverslips for the indicated period of time before processing for paxillin labeling or on vitronectin-coated dishes for 3 hours before recording images using an Axiovert inverted microscope (Zeiss) equipped with motorized stage, heated and CO₂-regulated incubator. Phase contrast images were taken every 15 minutes overnight using a x10/0.3 NA PH1 DIC1 objective and captured with a CCD MicroMax 1300 Y/HS camera (Roper Scientific) controlled by the Metamorph 7.0 software. Distance migration was calculated by manually tracking the nucleus of individual cells using the tracking module of Metamorph.

Statistical analyses

Box and whisker plots are used to describe the entire population without assumptions on the statistical distribution. They indicate 25th percentile (bottom line), median (middle line), 75th percentile (top line), 5th and 95th percentile (whiskers). For experiments with $n \geq 30$, statistical *t*-test was used to assess the statistical difference between two experimental conditions (Fig 8B and E) and analysis of variance in combination with a Dunnett post hoc-test was used to compare multiple conditions to the control (Fig.5B and C and Fig 8D). For experiments where $n < 30$ (Fig 7B, C, F and G), the non parametric Mann and Whitney test was used to assess the

statistical difference between two experimental conditions and the Kruskal and Wallis test was used to compare multiple conditions to the control. Multiple comparisons were performed according to Dunn's procedure. Statistical analyses were performed using Microsoft Excel and XLSTAT softwares (Addinsoft).

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FIGURE LEGENDS

Figure 1: RhoU is associated with the podosome belt in osteoclasts. RAW 264.7 cells were infected with retroviruses encoding for GFP (A) or GFP fused to RhoU WT (B), RhoU Q107L (C), or RhoU T63N (D). Cells were maintained in 25 ng/ml of RANKL for 5 days to obtain osteoclasts expressing the GFP fusion proteins. Osteoclasts were fixed and labeled for F-actin and vinculin. F-actin accumulates at the cell periphery together with vinculin forming a typical podosome belt (insets, inset a, for GFP-RhoU Q107L). GFP-RhoU WT and Q107L colocalized with vinculin, whereas GFP-RhoU T63N did not (insets). GFP-RhoU Q107L was also present around isolated podosomes (inset b). Bar, 50 μm . Insets are enlarged 2 times.

Figure 2: GFP-RhoU Q107L is associated with Src-induced podosomes. HeLa cells were co-transfected with activated c-Src (Src Y527F) and GFP-RhoU Q107L. Cells were fixed and labeled for Src (A) or vinculin (B) and F-actin (right hand panel). GFP-RhoU Q107L (middle panel) surrounds totally or partially the actin core of podosomes (arrowheads). Some actin dots, colocalized with c-Src (arrows in A), are devoid of RhoU Q107L (arrows in insets). Actin dots without RhoU Q107L are also devoid of vinculin (arrows in insets in B). Bar, 10 μm . Insets are enlarged 2 times.

Figure 3: GFP-RhoU is associated with FAs. HeLa cells were transfected with GFP-RhoU WT (A), GFP-RhoU Q107L (B) or GFP-RhoU T63N (C). Cells were labeled for paxillin (middle panel). Merging of RhoU (left panel) and paxillin staining is shown on the right panel. Bright spots out of focus are vesicles where GFP-RhoU accumulates. D and E show GFP-RhoU localization in FAs at the substratum (D) and in vesicles 2 μm above the substratum (E). Percentage of GFP-RhoU WT or GFP-RhoU Q107L expressing cells

localized to FAs is shown in F. Error bars indicate SD of 3 independent experiments. Bar, 10 μm .

Figure 4: The hypervariable region and effector binding domain are crucial for RhoU targeting to FAs. A, Schematic representation of constructs used in this study. B-G, HeLa cells transfected with GFP tagged- $\Delta\text{NRhoU Q107L}$ (B), $-\Delta\text{NRhoU Q107L}\Delta\text{C}$ (C), $-\Delta\text{NRhoU Q107L C255,256S}$ (D), $-\text{C-ter}$ (E), $-\text{RhoU Q107L 3M}$ (F) or the Cdc42-RhoU chimera (G) were labeled for paxillin (inset). Insets are reduced 3 times. H, Percentage of cells with GFP-RhoU mutants localized to FA. Error bars indicate SD. I, To study the binding properties of RhoU mutants, HeLa cells were transfected with the indicated constructs. Cell lysates were subjected to GST-CRIB pull down assays and analyzed by Western blot using an anti GFP antibody.

Figure 5: GFP-RhoU Q107L modifies FAs number and distribution in cells. A, Immunostaining for paxillin in NIH3T3 cells expressing either GFP alone or GFP-RhoU Q107L. Note the increase in cytoplasmic accumulation of paxillin in GFP-RhoU Q107L expressing cells. Bar 10 μm . B, Box-and-whisker diagrams describing the number of FAs in cells expressing GFP, GFP-RhoU WT, GFP-RhoU Q107L or GFP-RhoU T63N. C, Box-and-whisker diagrams describing the distribution of FAs from the periphery to the center of the cell as determined by Euclidean distance map (EDM). $n \geq 30$ cells for each point. Stars indicate significant differences with GFP control: * $p < 0,05$, *** $p < 0,001$, n.s.: not significantly different from control.

Figure 6: RhoU-dependent differences in FA formation and distribution in response to adhesion stimuli. A, NIH3T3 cells expressing GFP-RhoU Q107L were seeded for 15, 30 and

60 minutes on 5 µg/ml vitronectin and stained for paxillin. GFP-RhoU Q107L was excluded from peripheral adhesion structures (arrows) and colocalized with paxillin in more centripetally localized adhesions at 15 and 30 minutes (arrowheads). At 60 minutes, most adhesions are positive for both paxillin and GFP-RhoU Q107L (arrows). Insets are enlarged 2 times. Bar: 10 µm. B and C, number and distribution of FAs in NIH3T3 cells seeded on 5 µg/ml vitronectin for 60 minutes. Cells are stained for paxillin and processed as described in Materials and Methods. 20 < n < 30 cells for each point. D, Bar graph shows the cDNA levels determined by quantitative RT-PCR amplification of RhoU in cells expressing Luciferase (shLuc) or RhoU (shRhoU) shRNA that were used for the adhesion assay. Error bars: 95% confidence limits of the RhoU/Gapdh ratios. E, Immunostaining for paxillin in shLuc and shRhoU NIH3T3 cells seeded for 30 minutes on vitronectin. Bar, 10 µm. F and G, number and distribution of FAs in NIH3T3 cells seeded on 5 µg/ml vitronectin for 30 or 60 minutes. In B, C, F, G, stars indicate significant differences with GFP control or shLuc; * p < 0,05. n.s.: not significantly different from control.

Figure 7: RhoU affects cell migration. NIH3T3 cells expressing GFP, GFP-RhoU WT, GFP-RhoU Q107L or GFP-RhoU T63N were seeded on 5 µg/ml vitronectin for 3 hours. Cell movements were observed under a phase contrast microscope and recorded. A, Phase contrast images show the morphology of cells 8 hours after starting the recording. Bar 30 µm. Tracks of 10 cells that remained in the microscope field during the entire experiment (17,5 hours) are represented on the right panels. B, Box-and-whisker diagrams describing the distance covered by cells during the recording. C, shLuc or shRhoU NIH3T3 cells were processed and recorded as above. Cells were recorded for 20,5 hours. n ≥ 30 cells for each point. Stars indicate significant differences with GFP or shLuc controls: * p < 0,05, ** p < 0,01, n.s.: not significantly different from control.

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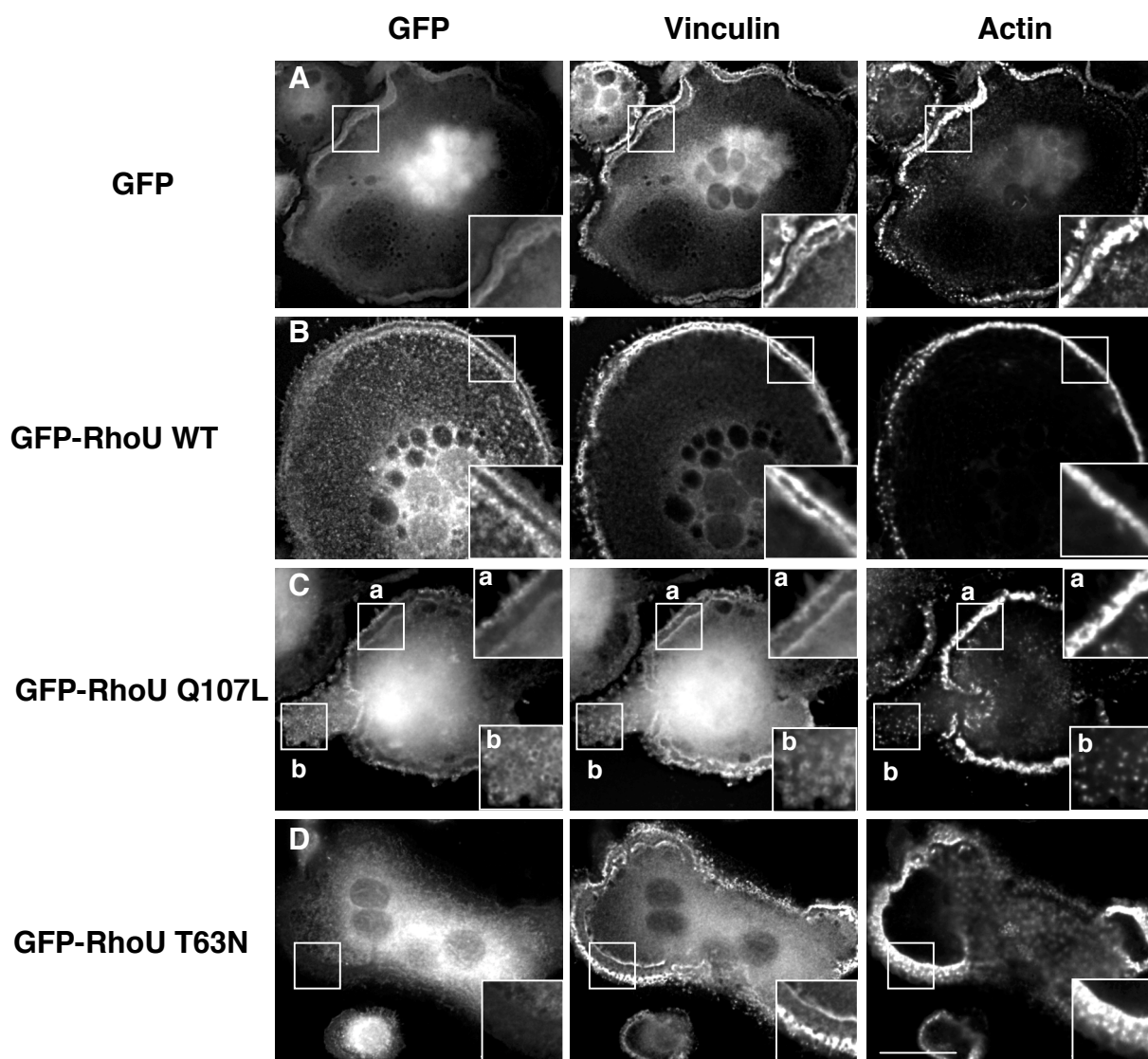
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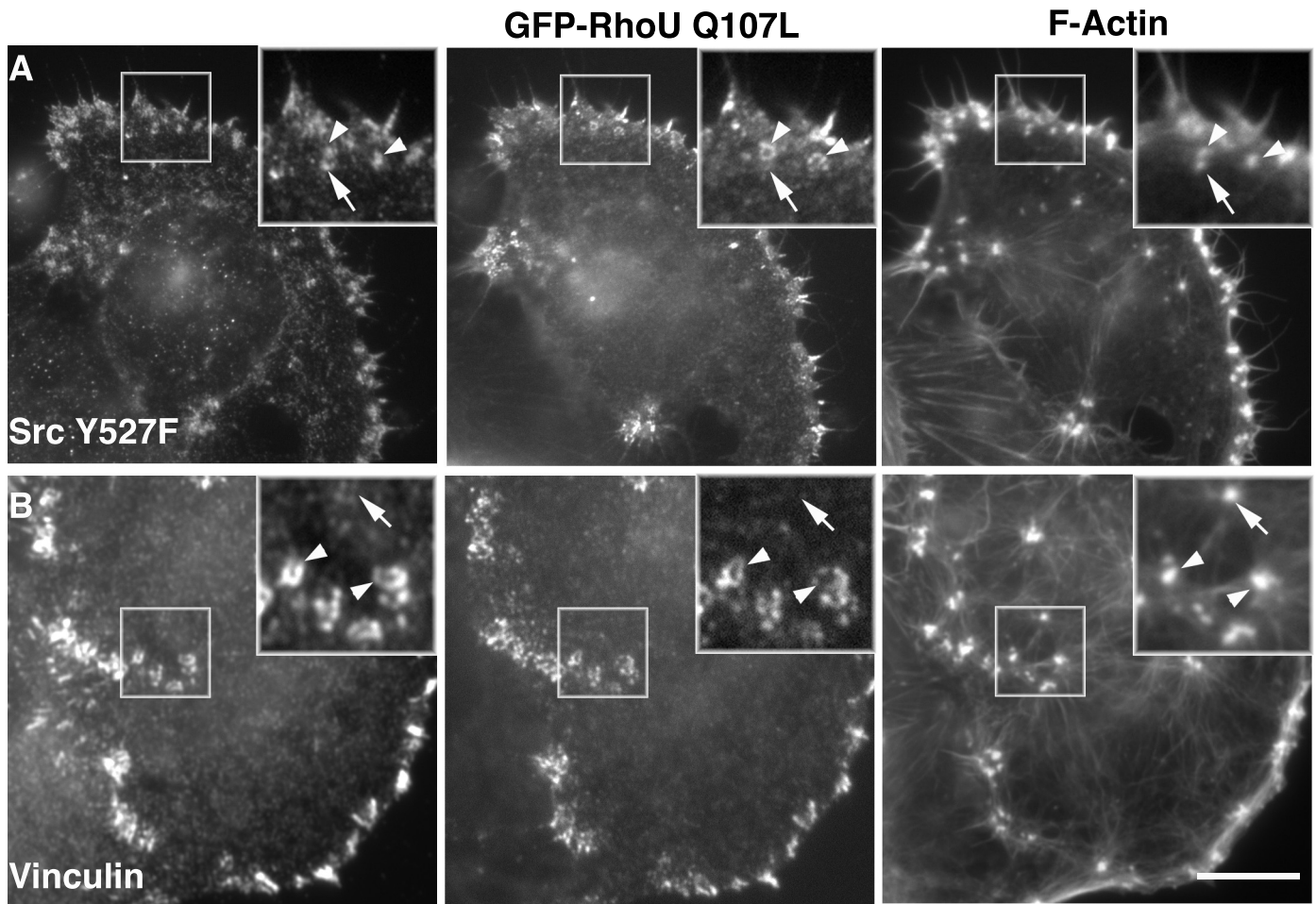
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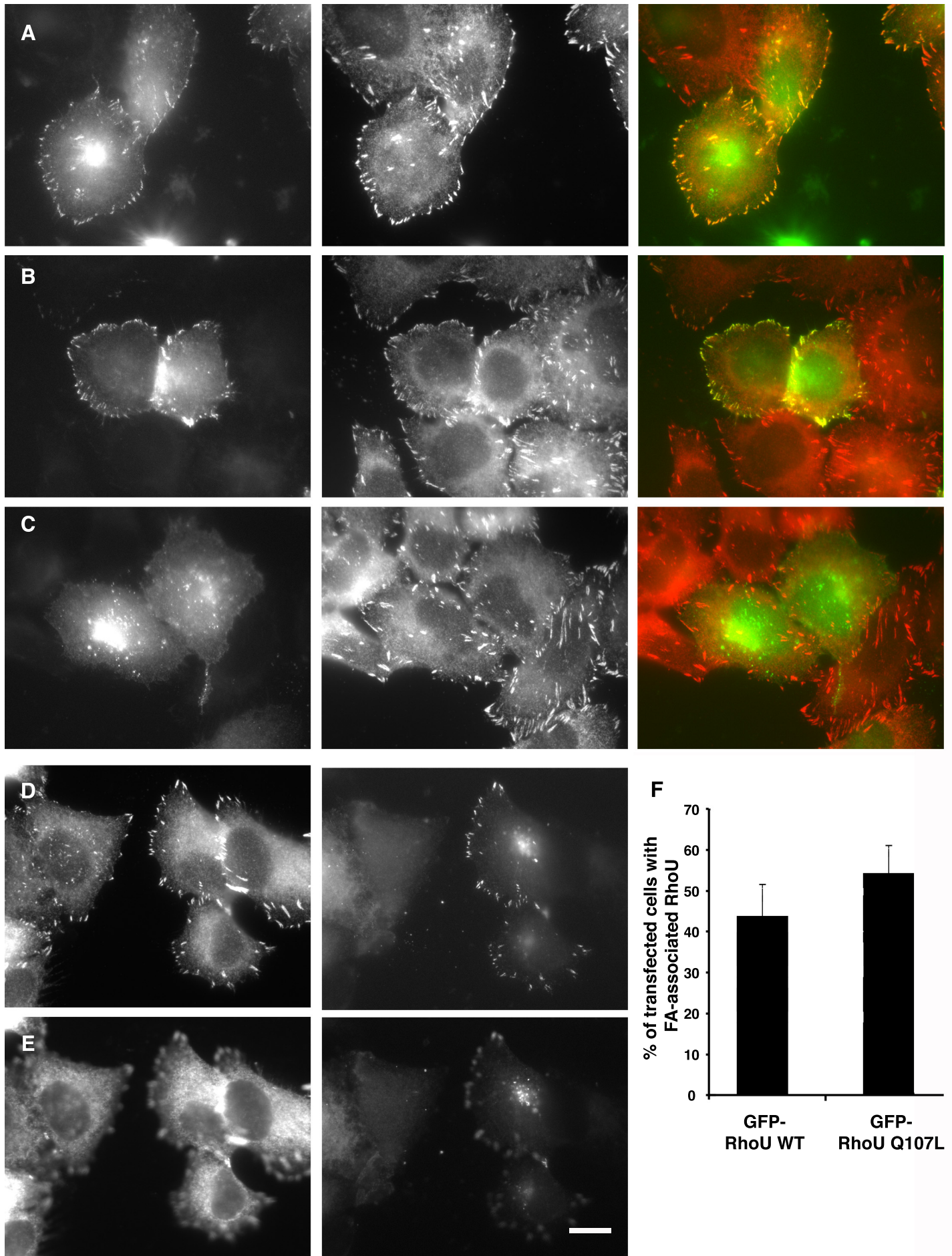
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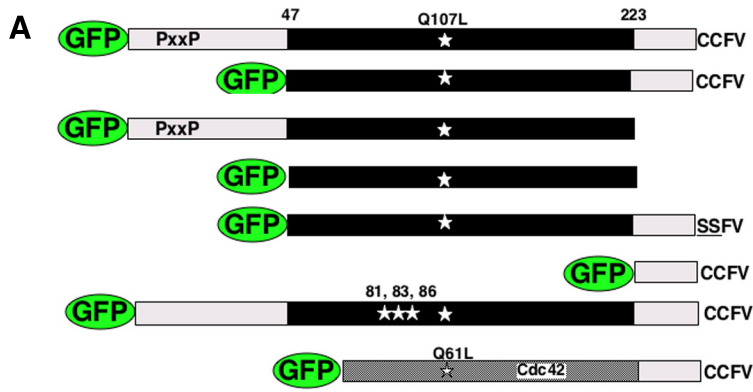
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- RhoU Q107L
 (B) Δ NRhoU Q107L
 RhoU Q107L Δ C
 (C) Δ NRhoU Q107L Δ C
 (D) Δ NRhoU Q107L C255,256S
 (E) C-ter
 (F) RhoU Q107L 3M
 (G) chimera

