UNC119a bridges the transmission of Fyn signals to Rab11, leading to the completion of cytokinesis

YuKyung Lee,1 Sunglan Chung,2 In Keol Baek,1 Tae H. Lee,1 Soon-Young Paik3,* and JooHun Lee1,*

1Department of Systems Biology; Yonsei University; Seoul, Korea; 2University College; Yonsei University; Seoul, Korea; 3Department of Microbiology; College of Medicine; The Catholic University; Seoul, Korea

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Abbreviations: UNC119, uncoordinated 119; SFKs, Src family protein tyrosine kinases; ERK, extracellular signal-regulated kinase; Ab, antibody; GFP, green fluorescent protein

Introduction

During cytokinesis, the final stage of cell division, a cell containing two duplicated nuclei physically separates into two daughter cells. This process requires the orchestrated interaction of various cellular activities, including signal transductions, the reorganization of the cytoskeleton and the transportation and fusion of membrane components to the dividing site (for a review, see refs. 1–4). Src family kinases (SFKs) transduce signals from the extracellular environment to intracellular signal transduction pathways and, hence, are involved in various cellular functions such as cell proliferation and differentiation, organization of the cytoskeleton, adhesion to the extracellular matrix, motility and migration (for a review, see refs. 5–6).

Several reports have suggested a possible role of SFK signaling in cytokinesis; however, the underlying mechanism remains to be elucidated.7,9 Recently, Kasahara et al.10 reported that SFK signaling leads to the phosphorylation and midbody localization of ERK, and that this process is required for the completion of cytokinesis in HeLa cells. In this report, the phosphorylation and midbody localization of ERK was shown to be Rab11-dependent.10 These analyses and previous reports addressing the role of Rab11 in cytokinesis11-14 suggest that SFK signaling is transmitted to Rab11 for the completion of cytokinesis.

Src family kinases (SFKs) regulate the completion of cytokinesis through signal transduction pathways that lead to the Rab11-dependent phosphorylation of ERK and its localization to the midbody of cytokinetic cells. We find that UNC119a, a known activator of SFKs, plays essential roles in this signaling pathway. UNC119a localizes to the centrosome in interphase cells and begins to translocate from the spindle pole to the spindle midzone after the onset of mitosis; it then localizes to the intercellular bridge in telophase cells and to the midbody in cytokinetic cells. We show that the midbody localization of UNC119a is dependent on Rab11, and that knocking down UNC119a inhibits the Rab11-dependent phosphorylation and midbody localization of ERK and cytokinesis. Moreover, we demonstrate that UNC119a interacts with a Src family kinase, Fyn and is required for the activation of this kinase. These results suggest that UNC119a plays a key role in the Fyn signal transduction pathway, which regulates the completion of cytokinesis via Rab11.
(3) UNC119a interacts with and activates Rab11. Based on this hypothesis, we tested whether UNC119a functions in the SFK signal transduction that leads to the completion of cytokinesis. In this paper, we report that UNC119a localizes to the midbody of dividing animal cells in a Rab11-dependent manner and plays essential roles in transmitting Fyn kinase signals to Rab11, suggesting that UNC119a plays an essential role in the completion of cytokinesis.

Results

UNC119a localizes to the midbody of cytokinetic cells. To examine the distribution of UNC119a in mammalian cells, we fixed HeLa cells with cold methanol and immunostained the cells with an anti-UNC119a antibody (UNC-Ab) raised against mouse UNC119a (Fig. 1; see “Materials and Methods” for details). In interphase cells, UNC119a was concentrated in one or two distinct spots. Double immunostaining of cells with UNC-Ab and monoclonal γ-tubulin-antibody (Ab) indicated that UNC119a was concentrated at the centrosomes (Fig. 1A).

In metaphase cells, UNC119a was mainly found at the spindle poles and in some of the spindle fibers. Many punctate UNC119a spots were also found throughout the cytoplasm. In early anaphase cells, UNC119a was concentrated at the spindle poles, as in metaphase cells, and at the spindle midzone in numerous spots. In late anaphase cells, UNC119a was not observed at the spindle poles but was concentrated at the intercellular bridge of the cells. In telophase and cytokinetic cells, UNC119a was localized at the intercellular bridge and midbody of the cells (Fig. 1A). Preincubation of UNC-Ab with GST (glutathione-s-transferase)-tagged UNC119a completely inhibited the binding of the Ab in the fixed cells, suggesting that UNC-Ab binding was specific (Fig. 1B). Western blotting experiments with cell extracts prepared at different stages of the cell cycle showed no significant changes in the cellular content of UNC119a during the cell cycle (Fig. 1C). This cell cycle-dependent localization of UNC119a was also observed in five different cell types fixed using the same method (three human cell lines, HEK293, U2OS and htrRPE-1; and two mouse cell lines, NIH3T3 and MEF) (Fig. S1).

As it has been reported that UNC119 is associated with membrane vesicles, we also examined the distribution of UNC119a in cells fixed using a different method that well preserved the staining of the membrane vesicle-associated protein, p230 (Fig. S2A). In these experiments, where cells were fixed with paraformaldehyde, permeabilized with Triton X-100 (see “Materials and Methods”) and then immunostained with UNC-Ab, we also observed that UNC119a was concentrated in different regions of the cells depending on the cell cycle stage (Fig. 1D).

Although the results of these two sets of experiments were basically identical, there were some minor, but reproducible, differences depending on the applied fixation method. In interphase cells fixed with cold methanol, UNC119a was distinctly concentrated at the centrosomes (Fig. 1A). However, in interphase cells fixed with paraformaldehyde, UNC119a was observed as many punctate spots in the cytoplasm and at the centrosomes (Fig. 1D). In metaphase cells fixed with paraformaldehyde, UNC119a was detected as numerous spots along spindle fibers. Although some of the UNC119a spots were concentrated at the spindle poles, the localization to the poles was not as distinct as it was in methanol-fixed cells (see metaphase cells in Fig. 1A and D). Concentration of UNC119a at the intercellular bridges of cytokinetic cells was evident in cells fixed with either method (Fig. 1A and D); however, concentration of UNC119a at the midbody was not observed in paraformaldehyde-fixed cells (Fig. 1D). Instead, there was a clear gap in the middle of the intercellular bridges, corresponding to the presumed site of the midbody (Fig. 1D). It has been reported that the midbody is surrounded by electron-dense material of an unknown nature, suggesting that the paraformaldehyde fixation and permeabilization procedure was not sufficient to allow UNC-Ab to penetrate the surrounding material. We obtained essentially identical immunostaining results when U2OS cells were fixed with paraformaldehyde and permeabilized as described above (data not shown). Alternatively, we observed that UNC119a was concentrated at centrosomes and midbodies when GFP-UNC119a was expressed in U2OS cells (Fig. S2B). These results showed that the localization of UNC119a is regulated during the cell cycle in these cells and suggested that this process is associated with vesicle-transport during the cell cycle.

Different regions of UNC119a are required for the cell cycle-regulated localization of UNC119a. To identify domains that function in the cell-cycle regulated localization of UNC119a, we constructed five deletion mutants of human UNC119a and expressed the GFP-tagged deletion mutants in U2OS cells (Fig. S3). We first identified domains involved in the centrosomal localization of UNC119a by fixing transfected cells with cold methanol 12 h after transfection and immunostaining the cells with monoclonal γ-tubulin-Ab. When the GFP-tagged C-terminal domain of UNC119a (amino acids 121–240, GFP-C) was expressed, concentration of GFP-C at the centrosome was observed in 73.3% of the cells expressing the fusion protein (Fig. S3). Centrosomal localization of a truncation mutant including amino acids 60–240 (GFP-MC) and that of wild type UNC119a (GFP-T) was observed in 39% and 30% of cells expressing the fusion proteins, respectively (Fig. S3).

Although western blotting results indicated that all GFP-tagged mutant UNC119a proteins were synthesized in similar amounts in the transfected cells (data not shown), the distribution of amino acids 1–59 (GFP-N) or that of amino acids 60–120 (GFP-M) was difficult to detect in cells fixed with cold methanol. These results suggested that the C-terminal half of UNC119 (amino acids 121–240) was required for the centrosomal localization of the protein and that the N-terminal half of UNC119a was likely associated with vesicles (see above).

To identify domains involved in the midbody localization of UNC119a, we incubated cells for 48 h after transfection to obtain a sufficient number of cells undergoing cell division and fixed the cells with paraformaldehyde as the above results suggested that the N-domain (amino acids 1–59) and M-domain (amino acids 60–120) of UNC119a were associated with vesicles. The cells were then immunostained with monoclonal α-tubulin-Ab, and the cytokinetic cells expressing GFP were scored. Concentration
Figure 1. Cell cycle-dependent localization of UNC119a. Asynchronously growing HeLa cells were fixed in cold methanol (A and B) or paraformaldehyde (D). Fixed cells were double immunostained with UNC-Ab and monoclonal α-tubulin-Ab (or monoclonal γ-tubulin-Ab). The binding of the antibodies was visualized using Alexa 546-conjugated goat anti-rabbit IgG (red) and Alexa 488-conjugated goat anti-mouse IgG (green), respectively. The nuclei and chromosomes were stained with DAPI. (A) Cell cycle-dependent localization of UNC119a. White arrows show regions of highly concentrated UNC119a. Bars: 5 μm, in interphase; 10 μm, in other images. (B) UNC-Ab specifically recognizes endogenous UNC119a. Fixed HeLa cells were double immunostained with monoclonal α-tubulin-Ab and UNC-Ab pre-incubated with GST-UNC119a (UNC + UNC Ag). Pre-incubation of UNC-Ab with the antigen completely inhibited the binding of UNC-Ab to the endogenous protein. Bars, 20 μm. (C) The cellular content of UNC119 does not change during the cell cycle. HeLa cells were synchronized at G1/S using a double thymidine block. Synchronized cells were released into fresh medium, collected at the indicated times and lysed with RIPA buffer. Western blotting was performed with three antibodies: UNC-Ab for UNC119a; anti-phospho-histone H3 (Ser 10)-Ab, pHH3(S10), as a mitosis marker; and monoclonal α-tubulin-Ab as a loading control. A.S., asynchronous cell extracts. (D) Cell cycle-dependent localization of UNC119a in paraformaldehyde-fixed cells. Asynchronously growing HeLa cells were fixed with 4% paraformaldehyde and permeabilized in 0.2% Triton X-100 in PBS. Double immunostaining was performed as described above. White arrows indicate the centrosomes. Bar, 20 μm.
of GFP-N and of GFP-M at the midbody was observed in 82.4% and 60% of the cells, respectively (Fig. S3). Fusion of the two domains (GFP-NM) had no additive effect on the midbody localization (53%). Only 6.3% of the cells showed midbody localization when GFP-C was expressed (Fig. S3). Together, these results suggested that amino acids 1–120 and 121–240 of UNC119a were each strongly involved in the midbody localization and centrosomal localization of UNC119a, and that the midbody localization of UNC119a might be related to vesicle transport.

**UNC119a is required for the completion of cytokinesis.** Because UNC119a was concentrated at the intercellular bridge and midbody in cytokinetic cells, we next tested whether UNC119a was involved in cytokinesis by treating HeLa cells with siRNAs targeting human UNC119a (Fig. 2). RT-PCR and western blotting experiments showed that the siRNA treatment effectively decreased the cellular concentration of UNC119a (Fig. 2A and B) but had no effect on the cellular concentration of UNC119b mRNA (Fig. 2B). To test the effect of UNC119a siRNA on cell division, siRNA-treated HeLa cells were fixed and double immunostained with UNC-Ab and monoclonal α-tubulin-Ab. UNC119a siRNA treatment significantly increased the percentages of bi-nucleated cells and cytokinetic cells containing a long intercellular bridge with a centrally located midbody (Fig. 2C). In control cells, the percentages of bi-nucleated cells and cytokinetic cells were approximately 4.47% and 2.36%, respectively, while in UNC119a siRNA-transfected cells, the percentages increased to 19.86% and 7.58%, respectively (Fig. 2D). The siRNA treatment had no detectable effect either on the organization of cytoplasmic microtubules in interphase cells or the formation of spindle fibers in mitotic cells (Fig. 2E). These results suggested that UNC119a is required for the completion of cytokinesis, but not for either the organization of cytoplasmic microtubules or the formation of spindle fibers. Identical results were obtained when similar experiments were performed in hRPE-1 cells (data not shown).

**UNC119a interacts with Rab11 in a cell cycle-dependent manner.** A tendency of bi-nucleated cells and cytokinetic cells that exhibit an elongated intercellular bridge with a midbody has been observed in cells that are defective in abscission.10,11,44-46 It has also been reported that Rab11, a small GTPase, is localized at the intercellular bridge and midbody in cytokinetic cells and functions in abscission via transporting vesicles to the midbody.11 Moreover, an interaction between Rab11 and UNC119a has been reported in the transportation of Lck, a member of the SFKs, to the plasma membrane during T-cell activation.32 Based on these observations, we examined the possible interaction of UNC119a with Rab11 in proliferating HeLa cells.

In interphase cells fixed with paraformaldehyde and double immunostained with monoclonal Rab11a-Ab and UNC-Ab, both Rab11a and UNC119a were found throughout the cytoplasm of the cells in numerous spots. However, co-localization of UNC119a and Rab11a at these spots was not evident (Fig. 3A). In anaphase cells, both Rab11a and UNC119a were found as punctate dots, and co-localization of the two proteins was observed in most of the spots. In telophase and cytokinetic cells, the two proteins were present at the intercellular bridge and the midbody (Fig. 3A). These results showed that UNC119a and Rab11a co-localize in dividing cells and suggested that the co-localization of the two proteins is a regulated process during the cell cycle.

To examine the possible cell cycle-dependent interaction between UNC119a and Rab11a, immunoprecipitation experiments were performed using UNC-Ab and HeLa cell extracts prepared from asynchronously growing cells, G2/M-arrested cells and cells undergoing cell division (“Materials and Methods”). Western blottings with Rab11a-Ab showed that Rab11a co-precipitated with UNC119a, and that there was a noticeable increase in the co-precipitated amount of Rab11a in the mitotic cell extract, suggesting that UNC119a and Rab11a specifically interacted in mitotic cells (Fig. 3B). The interaction between Rab11a and UNC119a was confirmed by immunoprecipitating Rab11a in mitotic cell extracts and western blotting this with UNC-Ab (Fig. S4A). These results supported the idea that UNC119a and Rab11a interact in a cell cycle-specific manner.

Western blotting with Rab4-Ab suggested a possible interaction between UNC119a and Rab4, especially in G2/M-arrested cells. However, this interaction was observed only when the film was exposed for a prolonged period (e.g., overnight). These results suggested that only a minute fraction of Rab4 interacts with UNC119a and that the Rab4-UNC119a interaction is regulated differently from Rab11a-UNC119a interaction. The interaction between UNC119a and Rab4 could not be confirmed by a reverse immunoprecipitation. Immunoprecipitation using the Rab4-Ab and western blotting the precipitated proteins with the Ab showed that this Ab did not precipitate Rab4 (data not shown).

**Midbody localization of UNC119a is dependent on Rab11a activity.** We next examined the localization of UNC119a in HeLa cells treated with Rab11a siRNA to test whether Rab11a functions in the cell cycle-dependent localization of UNC119a. Rab11a siRNA treatment drastically decreased the amount of Rab11a in the treated cells (Fig. 3C) and inhibited the completion of cytokinesis as determined by the increase in both the number of bi-nucleated cells41 and the number of cytokinetic cells with long intercellular bridges (Fig. 3D). Double immunostaining cells with monoclonal α-tubulin-Ab and UNC-Ab showed that this siRNA treatment also inhibited the localization of UNC119a to the intercellular bridge and the midbody of cytokinetic cells (Fig. 3E and F). The Rab11 siRNA treatment had no effect on the concentration of UNC119a either at the centrosome of interphase cells or at the spindle poles of mitotic cells (Fig. 3E). In contrast, UNC119a siRNA treatment had no effect on the localization of Rab11a either in the pericentrosomal region in interphase cells or at the intercellular bridge of cytokinetic cells (Fig. 3G). Taken together, these results suggested that the transport of UNC119a to the intercellular bridge and midbody, possibly via associated endosomes, is dependent on Rab11a activity and is required for the completion of cytokinesis. These results also suggested that Rab11a is not involved in the centrosomal concentration of UNC119a in interphase cells. Because human cells exhibit two Rab11 isoforms, Rab11a and Rab11b, which are 91% identical in their amino acid sequences, we further examined the distribution of UNC119a in HeLa cells treated with siRNAs targeting both
Rab11 isoforms. Even though the double-siRNA treatment effectively decreased the amounts of both Rab11 isoforms below the detection limit, the inhibitory effect of the double-siRNA treatment on the completion of cytokinesis and on the localization of UNC119a was almost identical to that of treatment with siRNA targeting Rab11a alone (Fig. S4B). These results suggested that Rab11a plays an important role in the midbody localization of UNC119a (see “Discussion”).

UNC119a is required for the phosphorylation and transport of ERK to the midbody. Because the above results showed that UNC119a interacts with Rab11a, we next examined whether this interaction plays a role in the Rab11-dependent phosphorylation and midbody localization of ERK during cytokinesis (Fig. 4). Western blotting experiments using UNC119a siRNA-treated HeLa cell extracts showed that depletion of UNC119a inhibited the phosphorylation of ERK1/2 but had no effect on the cellular concentrations of these proteins (Fig. 4A). In addition, the midbody localization of ERK in cytokinetic cells was abolished in UNC119a siRNA-transfected cells (Fig. 4B). Inhibition of ERK phosphorylation via UNC119a antisense RNA treatment was also reported by Gorska et al.30 during T cell activation. These results and previous reports showing the interaction of UNC119a with different SFKs 29-31 suggested that UNC119a might be involved in the SFK signaling, leading to Rab11-dependent ERK phosphorylation. To test this possibility, we treated HeLa cells with PP2, an SFK inhibitor that caused cytokinetic cells with long intercellular bridges to appear (Fig. 4C), as reported by Kasahara et al.10 As expected, when we immunostained these cells with UNC-Ab, we did not observe midbody concentration of UNC119a. These results further suggested that UNC119a is involved in the SFK signal transduction, leading to completion of cytokinesis.

UNC119a interacts with and activates Fyn. To identify the SFK that interacts with UNC119a and functions in cytokinesis,
we performed immunoprecipitation experiments using UNC-Ab and HeLa cell extracts prepared from asynchronously growing cells, G₂/M-arrested cells and mitotic cells (see “Materials and Methods”). Western blotting experiments showed that the kinases Fyn and Yes, but not Src, co-immunoprecipitated with UNC119a. This co-immunoprecipitation of Fyn and Yes with...
Our results suggested that UNC119a binds to and activates Fyn kinase after that activated Fyn-UNC119a complex interacts with Rab11a. To test this possibility, we performed immunoprecipitation experiments using Rab11a (a + b) siRNA-treated cells. We found that Fyn co-immunoprecipitated with UNC119a (Fig. 5D). The results indicated that Fyn-UNC119a interaction was independent of Rab11. We next examined the effect of Fyn knockdown on the midbody localization of UNC119a and on the phosphorylation and midbody localization of ERK. Fyn siRNA treatment increased the percentage of bi-nucleated cells, suggesting that cytokinesis was inhibited (Fig. 5E). Interestingly, depletion of Fyn did not induce the accumulation of cytokinetic cells with long intercellular bridges, as was observed in UNC119a (or Rab11) siRNA-treated cells, but instead significantly decreased the number of cytokinetic cells. Whereas approximately 2% of the cell population was usually
undergoing cytokinesis in control cells, only 72 out of more than 7,000 cells (~1%) were undergoing cytokinesis in the Fyn siRNA-treated cells. Localization of UNC119a at the midbody was not observed in the majority of these cells (75%; n = 72). Fyn siRNA treatment also inhibited the phosphorylation and midbody localization of ERK (Fig. 5F and G), though it did not have a noticeable effect on the interaction between UNC119a and Rab11a (data not shown). Fyn siRNA treatment had no effect on the concentration of UNC119a at centrosomes and spindle poles (data not shown). Together, these results suggested that the activity of Fyn, UNC119a, and Rab11a are necessary for the phosphorylation and midbody localization of ERK, even though we did not measure Fyn kinase activity in this process. These results also suggested that UNC119a interacts with Rab11 in a manner independent of Fyn activity.

Discussion

Distinct regions of UNC119a are required for the cell cycle-dependent localization of UNC119a. Our data suggested that the C-terminal domain (amino acids 121–240) of UNC119a is essential for the localization of UNC119a at the centrosome, and that the centrosomal localization of UNC119a is not dependent on Rab11a (Fig. S3). At present, we do not understand how UNC119a localizes to centrosomes. Although our data suggest that UNC119a is not required either for the organization of cytoplasmic microtubules or the formation of spindle fibers, the concentration of the protein at centrosomes implies that UNC119a may function in other cellular events that are not examined in our current study. In this regard, it is interesting that Naegleria UNC119, which lacks the N-terminal 60 amino acid of human UNC119a but shows 46% amino acid sequence identity to the C-terminal 3/4 of human UNC119a (amino acids 60–240), is concentrated at the basal body, though is not involved in cytokinesis (Lee, unpublished observation).

The N-terminal domain of UNC119a (amino acids 1–59) was clearly involved in midbody localization. Gorska et al. (2004) previously showed that this domain is essential for the activation of Fyn by UNC119a, and our current results demonstrate that the activity of Fyn is necessary for the midbody localization of UNC119a. Collectively, this implies that the N-terminal domain plays a key role in transmitting Fyn signals to Rab11a and in the Rab11a-mediated midbody localization of UNC119a (see below). However, it has not yet been determined whether this domain alone can function in mediating the transmission of Fyn signals to Rab11a for the completion of cytokinesis.
Figure 5. For figure legend, see page 1312.
The middle (M) domain of UNC119a (amino acids 60–120) was also capable of localizing to the midbody, suggesting this domain may interact with protein(s) other than Fyn and form a protein complex distinct from the Fyn-UNC119a complex. These different complexes may interact with either a common protein, Rab11a, or with distinct proteins for the midbody localization.

Simultaneous binding of two distinct proteins to the N- and M-domains of one UNC119a molecule might have a negative effect on the midbody localization of UNC119a, resulting in the less efficient midbody localization of GFP-NM (amino acids 1–120) than that of either GFP-N or GFP-M (Fig. S3). Wild type UNC119a fused to GFP (GFP-T) also showed weaker centrosomal localization (30%) and midbody localization (51.7%) compared with the centrosomal localization of GFP-C (73.3%) and to the midbody localization of GFP-N (82.4%). These data suggest that overexpression of UNC119a and, hence, unregulated and/or simultaneous binding of different proteins to the respective binding sites in UNC119a inhibits the regulated localization of UNC119a, and that UNC119a is a multi-functional protein that interacts with diverse proteins. Indeed, in addition to the interaction of UNC119a with different SFKs via the N-terminal SH3-binding motif, interactions of UNC119a with various other proteins have been reported. UNC119a interacts with Abl kinase and dynamin in NIH3T3 cells, RIBEYE in photoreceptor ribbon synapses and transducin α in C. elegans sensory neurons. Interaction of UNC119 with different myristoylated proteins has also been reported.

Possible function of UNC119a in cytokinesis. Our results showed that UNC119a interacts sequentially with Fyn and Rab11a and is necessary for the completion of cytokinesis, suggesting that UNC119a plays important roles in mediating SFK signals for the completion of cytokinesis. However, there are several important points to be explored to fully understand the role of UNC119a in mediating SFK signals for the completion of cytokinesis.

If Fyn is the SFK that functions in regulating cytokinesis by interacting with UNC119a, it is expected that the depletion of Fyn and of UNC119a might have a similar inhibitory effect on the completion of cytokinesis. However, our results showed that depletion of UNC119a (Fig. 2D) is significantly more effective in inhibiting cytokinesis than depletion of Fyn (Fig. 5E). Our data also showed that the phenotype of UNC119a siRNA-treated cells (Fig. 2) and cells treated with PP2 treatment (Fig. 4C) is different from that of Fyn siRNA-treated cells. These results suggest the presence of other SFKs that are involved in the regulation of cytokinesis by interacting with UNC119a. We found that both Fyn and Yes interact with UNC119a, and that the depletion of UNC119a inhibits the activation of both kinases. However, we focused our study on Fyn, because the depletion of UNC119a had a more significant inhibitory effect on the activation of Fyn than on the activation of Yes. Hence, future studies addressing the role of Yes and possibly other SFKs will be needed to fully elucidate the role of SFKs in the completion of cytokinesis. Localization of Yes at the midbody of cytokinetic cells has recently been reported.

Similarly, the depletion of UNC119a was significantly more effective in inhibiting cytokinesis than the depletion of Rab11 (Figs. 2D and 3D). As discussed above, our data suggested that N-domain and M-domain of UNC119a can independently mediate the midbody localization of the protein. Our data also showed that the depletion of Rab11 was very effective but not complete in inhibiting the midbody localization of UNC119a. Taken together, our results suggest that, even though Rab11 might be the major protein that interacts with UNC119a to mediate SFK signals for the completion of cytokinesis, there are possibly other proteins that interact with UNC119a to mediate SFK signals for the completion of cytokinesis. Future studies on proteins that interact with UNC119a in cell cycle-dependent manner are necessary to fully understand the role of UNC119a in cytokinesis.
In addition, we demonstrated that knockdown of Rab11a alone or of both Rab11 isoforms in HeLa cells inhibited the completion of cytokinesis in the cells to a similar degree. One simple explanation for this finding is that Rab11a is important for the completion of cytokinesis, but Rab11b is not. Indeed, several reports have suggested that Rab11a and Rab11b play distinct roles. However, if Rab11a and Rab11b play different, but necessary, roles in a common pathway leading to the completion of cytokinesis, knockdown of either Rab11a alone or both Rab11 isoforms would have a similar inhibitory effect. Further studies are needed to clarify these two possibilities.

Human and mouse cells exhibit two UNC119 genes that encode two different proteins, UNC119a and UNC119b, which are approximately 60% identical in their amino acid sequences. However, a comparison of the amino acid sequences of the two proteins shows that the shared amino acid sequence identity between the two proteins mainly occurs in the C-terminal half of the proteins (amino acids 121–240), and that the SH3-binding motif, which is required for the interaction with SFKs, is not present in UNC119b. Recently, different localization patterns of the two UNC119 isoforms were also reported (UNC119a, centrosome; UNC119b, basal body and the primary cilium). These data suggest that UNC119a is the major protein functioning in SFK signal transduction leading to the completion of cytokinesis; however, this matter should be more definitively determined in future studies.

**Materials and Methods**

**Production of the UNC119 antibody.** We cloned the full-length mouse UNC119a cDNA, synthesized the protein in *E. coli* as an MBP-fusion protein, and raised a polyclonal antiserum by injecting the bacterially synthesized MBP-UNC119a into rabbits. The obtained polyclonal anti-UNC119a antibody (UNC-Ab) recognized both human and mouse UNC119a, which are 91% identical in their amino acid sequences. Western blotting experiments showed that the Ab recognized a human protein with a relative molecular mass of 36 kDa and a mouse protein with a relative molecular mass of 34 kDa, despite the fact that the calculated molecular weights of the two proteins are almost identical (26.99 kDa and 26.95 kDa, respectively) (Fig. S5B). The nature of this difference between the relative molecular masses of the two proteins was not clear. Pre-incubation of UNC-Ab with GST-tagged UNC119a completely inhibited the binding of the Ab to the presumed UNC119a proteins in each cell extract (Fig. S5B). Additionally, the Ab bound more intensely to the mouse protein than to the human protein in a reproducible manner, which could be due to mouse cells containing more UNC119a than the human cells or the Ab showing a stronger affinity for mouse UNC119a than for human UNC119a (Fig. S5B).

**Cell culture and cell cycle synchronization.** HeLa cells and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin (Hyclone). For synchronization at G/S, HeLa cells were cultured in the presence of 2 mM thymidine (Sigma) for 17 h, washed three times with PBS and cultured in fresh medium for 9 h. After a second thymidine treatment for 16 h, the cells were washed with PBS and released into fresh medium. To prepare G/M cell extracts, HeLa cells were cultured in the presence of nocodazole (40 ng/ml) for 12 h and harvested. To prepare mitotic cell extracts, HeLa cells were cultured in the presence of nocodazole, as above, and round-shaped cells were collected using “the mitotic shake-off” procedure. The cells were then washed with PBS, incubated in fresh medium for 1 h 45 min and harvested. This harvest time was determined by immunostaining a small portion of the cells with an α-tubulin Ab at 15 min intervals during the incubation.

**Plasmids, siRNAs and transient transfection.** Full-length and truncation mutants of human UNC119 cDNAs were subcloned into the pEGFP-C1 vector (Clontech). U2OS cells were transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. siRNA oligonucleotides that were designed and targeted to human UNC119a mRNA were purchased from GenePharma. The dsRNA oligonucleotides were as follows: Oligo-1, (sense) 5'-CAG AAG AAC GGU UGC CCA UCA ATT-3', (antisense) 5'-UUG AUG GGC AAC CGU UCU GAG-3'; Oligo-2, (sense) 5'-GGU UUA AGA UUC GGG ACA UTI-3', (anti-sense) 5'-UAG UCC CGA AUC UAA AAC CTC-3'; Oligo-3 (sense) 5'-CGU AGU AGA CCC AGU CUG ATT-3', (antisense) 5'-UCG AGG UGG GUC UCA UAC GGG-3'. Negative control dsRNA oligonucleotide: (sense) 5'-UUC UCC GAA CGU GUC ACG UTT-3', (anti-sense) 5'-AGC UGA CAC GUU CGG AGA ATT-3'. siRNA oligonucleotides targeting human Rab11 were designed as described by Wilson et al. and purchased from GenePharma. Fyn siRNA was purchased from Santa Cruz Biotechnology (sc-29321). HeLa cells were transfected with siRNA duplexes (30 nM) using Lipofectamine 2000 for 48–72 h according to the manufacturer’s instructions.

**Chemical.** PP2 was purchased from Calbiochem (529573). HeLa cells were treated with 10 μM PP2 in DMSO or DMSO alone (control) for 24 h. The cells were then fixed and immunostained with UNC-Ab.

**Antibodies.** An anti-UNC119 antibody (UNC-Ab) was generated as described above. The antibodies used in this study and the suppliers are listed below. Monoclonal anti-α-tubulin-Ab (Santa Cruz Biotechnology, sc-23948), monoclonal anti-Rab11a-Ab (Abcam, ab78337), monoclonal anti-γ-tubulin-Ab (Sigma, T6557), polyclonal anti-γ-tubulin-Ab (Sigma, T3559), monoclonal anti-p230 TGN-Ab (BD Transduction, 611281), polyclonal anti-ERK2-Ab (C-14, Santa Cruz Biotechnology, sc-154), polyclonal anti-phospho-p44/42 MAPK (ERK1/2) (Th2002/Tyr204)-Ab (Cell Signaling, #9101), polyclonal anti-phospho-histone H3 (Ser 10)-Ab (Millipore, #06-570), monoclonal anti-Fyn-Ab (FYN-01, Santa Cruz, sc-51598), monoclonal anti-Src [pY418]-Ab (phospho-Src: Invitrogen, AHO0051), polyclonal anti-Src-Ab (N-16, Santa Cruz, sc-19), monoclonal c-Yes-Ab (BD Transduction, 610376), polyclonal anti-Rab4-Ab (Cell Signaling, #2167), Alexa 488-conjugated goat anti-mouse IgG (Invitrogen, A11001) and Alexa 546-conjugated goat anti-rabbit IgG (Invitrogen, A11010).
Cell fixation, immunocytochemistry and confocal microscopy. To perform cold methanol fixation, cells were incubated in −20°C methanol for 5 min and washed with PBS. For paraformaldehyde fixation, cells were fixed with 4% paraformaldehyde in PBS for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 10 min at room temperature, then washed with PBS. For immunocytochemistry, cells were incubated in blocking solution (5% normal goat serum in PBS) for 1 h and then incubated with the primary antibody in PBS for 1 h 30 min at room temperature. After washing with PBS-T (PBS containing 0.1% Tween-20), the cells were incubated with a second antibody conjugated with a fluorescent dye for 1 h at room temperature. The cells were mounted on slides with Vectashield mounting medium (Vector Laboratories), and the samples were observed under an LSM 510 META confocal laser scanning microscope equipped with epi-fluorescence and a digital image analyzer (Carl Zeiss). Z-stacked images were acquired at 400× magnification.

**Immunoprecipitation.** Cells were collected and lysed in IP lysis buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100) containing protease inhibitors (5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A) and phosphatase inhibitors (1 mM sodium orthovanadate and 5 mM sodium fluoride). The cell lysates were preclarified with equilibrated protein A Sepharose beads (Amersham) for 1 h. After centrifugation, the supernatant was incubated with 1~2 μg of the appropriate antibody or preimmune serum overnight at 4°C and then incubated with equilibrated protein A sepharose beads for 1 h at 4°C. The beads were collected via centrifugation, washed three times with IP washing buffer (50 mM TRIS-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100) and resuspended in 1× SDS protein sample buffer. Proteins were separated using SDS-PAGE for western blotting analysis.

**Western blotting.** Cells were lysed in RIPA buffer (50 mM TRIS-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl; 1 mM EDTA, 0.1% SDS) containing protease inhibitors. The protein concentration of each cell lysate was measured via the Bradford assay, and an equal amount of protein from each sample extract was separated using SDS-PAGE and transferred to a nitrocellulose membrane.

**Statistical analyses of data.** Data are presented as mean ± standard deviation (S.D.) from at least three independent experiments. Significant differences between groups were determined by Student’s t-tests. Values of ***p < 0.001, **p < 0.01 and *p < 0.05 were considered statistically significant.

**Disclosure of Potential Conflicts of Interest** No potential conflicts of interest were disclosed.

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**Supplemental Materials** Supplemental materials may be found here: www.landesbioscience.com/journals/cc/article/24404

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