RLIP, an Effector of the Ral GTPases, Is a Platform for Cdk1 to Phosphorylate Epsin during the Switch Off of Endocytosis in Mitosis*

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The Ral signaling pathway is critically involved in Ras-dependent oncogenesis. One of its key actors, RLIP/RalBP1, which participates in receptor endocytosis during interphase, is also involved in mitotic processes when endocytosis is switched off. During mitosis, RLIP76 is located on the duplicated centrosomes and is required for their proper separation and movement to the poles. We have looked for actors that associate with RLIP during mitosis. We show here that RLIP/RalBP1 interacts with an active p34cdcd2 cyclin B1 (cdk1) enzyme and that this interaction is crucial for the mitotic phosphorylation of Epsin that, once phosphorylated, is no longer competent for endocytosis. We show also that this latter phosphorylation is dependent on Ral signaling. We propose that RLIP/RalBP1 is used as a platform by the mitotic cdk1 to facilitate the phosphorylation of Epsin, which makes Epsin incompetent for endocytosis during mitosis, when endocytosis is switched off.

In physiological signaling as well as in oncogenic transformation, the Ral GTPase is an effector of Ras and is required for transformation (1–3), cell migration (4), and metastasis (5). Downstream of Ral, two proteins have been identified as Ral effectors and involve the Ral pathway in trafficking, Sec5 and RLIP76/RalBP1. Sec5 mediates Ral contribution to functioning of the exocyst, and this interaction is critical for basolateral targeting of membrane proteins in polarized cells (6, 7). The RLIP76/RalBP1 protein interacts with the POBI/REPS proteins and the adaptin complex AP2, and these latter proteins bind Epsin. These interactions mediate the role of Ral in endocytosis (8, 9). During mitosis, a period of the cell cycle devoid of endocytosis (10), RLIP76, also known as cytokeratin, is found at the centrosome and on spindle microtubules, where it fulfills essential functions, because depletion of RLIP76 leads to monopolar spindles and mitotic catastrophes (11). The partners of mitotic RLIP76 are not known (11). Thus RLIP76 appears as a protein with two faces, one during interphase, one during mitosis, with no functional relationship between these two roles.

Pathways downstream of the Ral pathways are conserved in mammals, flies, probably Caenorhabditis elegans, and possibly in all Metazoans. All of the human proteins involved in the pathways downstream and upstream of Ral have an ortholog in Drosophila melanogaster, and these orthologs are networked according to a similar circuitry (12) where α-RLIP is the fly ortholog of human RLIP76/RalBP1 (9).

Looking for partners of RLIP proteins, we have screened human placenta and Drosophila embryo two-hybrid cDNA libraries with human and fly RLIP as baits, respectively. We show that cyclin B and RLIP interact in vitro as well as in vivo in both species and that RLIP associates with an active cyclin B2/p34cdk2 (cdk1)7 complex.

RLIP76 is found in a complex with the endocytic proteins Numb and Epsin, and this complex, as well as the complex between RLIP76 and AP2, is present both in interphase and during mitosis. Epsin has been shown to be phosphorylated by cdk1 in M phase, which might be related to the absence of endocytosis during mitosis, and this phosphorylation renders Epsin incompetent as a dominant negative molecule on endocytosis (13). We report that RLIP76 interacts in HeLa cells with both the phosphorylated and the non-phosphorylated forms of Epsin and that overexpression of the C terminus of RLIP blocks the phosphorylation of Epsin during mitosis.

We propose that, at the onset of G2/M, RLIP proteins function as scaffolds between its interphase endocytotic partners, including Epsin, and its G2/M partner cdk1. It would correspond to a sensitizer function for enzyme-substrate reaction and would be related to the switching off of endocytosis during mitosis. Surprisingly, blocking Ral signaling has a similar effect on Epsin phosphorylation, which suggests a role for Ral also at the approach of mitosis.

MATERIALS AND METHODS

Plasmids—For expression of the last 159 amino acids of RLIP76, the coding sequence was amplified using primers ggaggatccctgcatcgctggcagctt (forward) and cggatcatggctggctcctt (reverse) (Invitrogen) and cloned between the BamHI and EcoRI sites of pRK5-Myc.

Epsin expression plasmid was a gift from Dr. Di Fiore. Myc-tagged Ral binding domain expression plasmids have been described in a previous study (6). Myc-tagged full-length RLIP76 expression plasmids were generated by subcloning RLIP76 coding sequence in-frame with the myc tag expression region of pCS5-MT (a gift of Dr. D. Turner).

Cells and Transfections—HeLa cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum at 37 °C in 5% CO2. Schneider S2 cells were cultured in Schneider medium (Invitrogen) supplemented with 16.5 ma 1-glutamine (Sigma) and 10% fetal bovine serum.

HeLa cells were transiently transfected using the calcium phosphate method (14). For synchronization, exponentially growing HeLa cells were treated for 12–14 h with nocodazole (5 μg/ml), and plates were

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RLIP76 Brings p34cdc2-cyclinB1 to Epsin

FIG. 1. Pull-down with HeLa cell extracts and GST and GST-RLIP76. Exponentially growing HeLa cells (E) or HeLa cells shaken off after incubation with nocodazole (5 μM) for 12 h (M) to enrich in mitotic cells were harvested and lysed with Triton X-100 1% buffer. 200 μg of extract was incubated on beads with GST and GST-RLIP76 (6 nm) for 1 h at 4 °C. After extensive washing with lysis buffer, beads were boiled in Laemmli buffer, run on SDS-PAGE gels, and Western-blotted. Immunodetection was performed with anti-cyclinB1 antibodies (Transduction Laboratories).

| Pull-down          | Cell extracts | GST-RLIP76 |
|--------------------|---------------|------------|
|                    | M | E | M | E | M | E |
| CyclinB1           |   |   |   |   |   |   |

A Two-hybrid Screen Identifies CyclinB as a Specific Partner of RLIP in Drosophila melanogaster and Homo sapiens—Looking for partners specific of regions of RLIP proteins, we used the last 144 amino acids (of 625 amino acids) of ρ-RLIP as bait in a two-hybrid screen; among 5 million screened colonies, 11 His” β-galactosidase” clones corresponded to cyclinB. We did not identify cyclinB as a prey in any of eight other two-hybrid screens performed on the same library with irrelevant bait proteins, i.e. among 80 millions clones surveyed in various two-hybrid screens. The specificity of the RLIP-cyclinB interaction was further strengthened by the lack of interaction between cyclinB as a prey and various bait proteins (Ral, Ras, RGL, and lamin) (data not shown). CyclinB is composed of 787 amino acids, and all 11 selected clones encoded the GAL4-activation domain fused in-frame with the last 514 amino acids of cyclinB, starting just before the cyclin boxes that bind p34cdc2 (data not shown).

We tested whether this interaction is conserved in humans. CyclinB1 was tested in a two-hybrid assay with full-length RLIP76 (the C terminus of RLIP76 autoactivates and therefore cannot be used as a 2H bait) as well as with irrelevant proteins. The results showed that the interaction between cyclinB and RLIP is conserved between Drosophila and humans (data not shown).

CyclinB1 Interacts with RLIP76 in Vitro—HeLa cell extracts were prepared either from exponentially growing cells (“E” in Fig. 1) or from a population of cells enriched in mitotic cells (“M” in Fig. 1) by mechanical shake-off of cells pre-treated with nocodazole. Western blot analysis of whole cell extracts showed an increase of cyclinB1 between the exponentially growing cells (E) and the mitotic cells (M), validating that the latter population is indeed largely enriched in mitotic cells (Fig. 1). These cell extracts were incubated with GST or with GST-RLIP76 proteins bound to glutathione beads. Proteins bound to the column were analyzed by Western blot with anti-cyclinB1 antibodies. Fig. 1 shows that cyclinB1 binds specifically to GST-RLIP76.

CyclinB1 and RLIP76 Interact in Vivo—To assess whether the two proteins actually interact in vivo, we submitted protein extracts from exponentially growing cells (E) and from mitotic cells (M) to immunoprecipitation with anti-RLIP76 antibodies, and the immunoprecipitates were analyzed for the presence of cyclinB1 by Western blotting. Fig. 2A shows that cyclinB1 co-immunoprecipitates with RLIP76, whereas non-immune rabbit IgG used as a negative control precipitated neither RLIP76 nor cyclinB1. Conversely, immunoprecipitation with anti-cyclinB1 antibodies could not lead to detect RLIP76 in the immunoprecipitate, probably because only a fraction of cyclinB1 is complexed to RLIP76. To further validate the specificity of this immunoprecipitation, the serum was preincubated with GST or GST-RLIP76 (the anti-RLIP76 serum was obtained by immunizing rabbits with an MBP-RLIP76 fusion protein (9)). Preincubation with GST-RLIP considerably reduced the amount of immunoprecipitated cyclinB1, as opposed to preincubation with GST alone (Fig. 2B, upper panel).

Two-hybrid assays had suggested that the interaction between RLIP and cyclinB is conserved in flies and human. We tested this conservation using cell extracts from exponentially growing Schneider cells. Whereas pre-immune serum used as a control immunoprecipitated neither ρ-RLIP nor fly cyclinB, antibodies raised against ρ-RLIP immunoprecipitated cyclinB together with n-RLIP (Fig. 2C), hence confirming in vivo the conservation of this interaction.

RLIP Is Associated with an Active Cdk1 Complex

Because cyclinB1 is a component of the cdk1 kinase holoenzyme, we tested whether RLIP76 interacts with cyclinB1 alone or complexed to p34cdc2. Cdk1 activity is assessed conventionally by the in vitro phosphorylation of histone H1 and is maximal in mitotic cell extracts (16). Cell extracts prepared from exponentially growing cells and mechanically shaken mitotic cells were incubated with beads bound to p13, GST, or GST-RLIP76. p13 binds cyclinB and constitutes a positive control (Fig. 3). No H1 kinase activity was pulled-down from exponentially growing cell extracts either with GST-RLIP76 or with p13. As opposed to GST, GST-RLIP76 pulled down a strong H1 kinase activity from mitotic cell extracts (Fig. 3). This activity was also immunoprecipitated, only from mitotic cell extracts, with anti-RLIP76 antibodies (Fig. 2B, lower panel), and its level was considerably lower when the serum had been depleted by incubation with GST-RLIP but not with GST (Fig. 2B, lower panel). All together, these results show that RLIP76 associates with the active cdk1 holoenzyme. Finally, we veri-
Table 1. Immunoprecipitation of Cyclin-B1 and RLIP76

| Protein | RLIP76 | Cyclin-B1 | IgG |
|---------|--------|-----------|-----|
| E       | ![RLIP76](image) | ![Cyclin-B1](image) | ![IgG](image) |
| M       | ![RLIP76](image) | ![Cyclin-B1](image) | ![IgG](image) |

Fig. 2. RLIP and cyclinB interact in vivo. A, human RLIP76 and cyclinB are found in a complex in vivo. Cell extracts (200 μg) prepared from exponentially growing cells (E) or from mitotic cells (M) were immunoprecipitated with anti-RLIP76 antiserum (9) or 10 μg of rabbit IgG. After electrophoresis and Western blotting, proteins in the immunoprecipitate were revealed with anti-RalBP1 antibodies (Santa Cruz Biotechnologies) and with anti-cyclinB1. B, the same experiment as in A was performed with extracts from M cells except that anti-RLIP76 serum was preincubated with GST fused to RLIP76 (upper panel). The same immunoprecipitates were tested for the presence of an H1 kinase activity (lower panel). C, fly RLIP interacts with fly cyclinB in vivo. Cell extracts from exponentially growing Schneider cells were immunoprecipitated with serum raised against fly RLIP or with pre-immune serum (pi). After electrophoresis and Western blotting, immunodetection was performed with the same anti-RLIP76 serum and with anti-DeyclinB antibodies (a gift from Dr. P. Leopold).

RLIP76 Participates in Endocytotic Complex(es), with AP2, Numb, and Epsin throughout the Cell Cycle in HeLa Cells

We questioned the fate of endocytotic complexes containing RLIP76 in mitosis when RLIP76 interacts with cdk1. RLIP76 forms a complex with AP2 and POB1/REPS, and these latter proteins interact with Epsin (9, 13, 18). AP2 and Eps15 interact with each other as well as with Numb, a protein involved in endocytosis and in asymmetric division of Drosophila neuroblasts (19, 20). RLIP was reported to be in a complex with Epsin, and Fig. 4A shows that both AP2 and Numb were co-immunoprecipitated with RLIP76. Preincubating the anti-RLIP serum with GST-RLIP76 decreased the level of both RLIP76 and Numb in the immunoprecipitate (data not shown), as it had been shown for AP2 (9). siRNAs against RLIP76 efficiently knocked-down the amount of RLIP76 without affecting the level of AP2 and Numb in whole cell extracts (Fig. 4A).

In HeLa cells transfected with siRLIP76, immunoprecipitation with anti-RLIP76 antibodies showed a strong decrease of Numb and AP2 in the “immunoprecipitate” (Fig. 4A). This interaction between RLIP and Numb is also conserved between human and flies, as is the interaction between RLIP and mut2, the medium chain of the AP2 complex (9) (Fig. 4B).

Although RLIP is involved in endocytosis, it has its own function during mitosis. We investigated whether endocytotic complexes containing RLIP76 remained present during mitosis. Fig. 4C shows that, although there is a smaller amount of RLIP76 in whole cell extracts from mitotic cells, Numb and AP2 were found in RLIP76 immunoprecipitates, showing that complexes between RLIP76 and Numb, as well as between RLIP76 and AP2, are cell cycle-independent.

Epsin associates with POB1 and AP2 and is a substrate of the p34cdc2 kinase (13, 21). We questioned whether RLIP76, via its interaction with cyclinB, was critical for the mitotic phosphorylation of Epsin by cdk1. In exponentially growing cells, Epsin was present in an anti-RLIP76 immunoprecipitate, but during mitosis, when less RLIP was immunoprecipitated, Epsin could barely be detected (Fig. 4C). Yet both proteins were actually able to interact in mitotic cells, as evidenced by their co-immunoprecipitation from exponentially growing as well as mitotic cells that have been transiently transfected with expression vectors encoding RLIP76 and Epsin (Fig. 5). Moreover, as expected, in mitotic cells, Epsin was found present as two species with the slower migrating form resulting from its phosphorylation by p34cdc2 (13), and both were co-immunoprecipitated with RLIP. In ex vivo experiments, the affinity between AP2 and Epsin is decreased when Epsin is phosphorylated by p34cdc2 from Xenopus or CHO mitotic cell extracts (13, 21). In pull-down experiments with CHO cell extracts, the affinity of phosphorylated Epsin for POB1 is also decreased (13). An inference of these results would be that Epsin should display an lower interaction with RLIP during mitosis than during interphase. However, Fig. 5 shows that in HeLa cells RLIP76 interacts similarly with cdk1-phosphorylated and non-phosphorylated Epsin. We investigated also whether mitotic HeLa cells also displayed a reduced interaction between AP2 and Epsin as compared with exponentially growing cells. As shown in Fig. 6, all of the endogenous Epsin from mitotic cells exhibited the mobility shift characteristic of its phosphorylation by p34cdc2;
yet similar levels of AP2 were found in Epsin immunoprecipitates from mitotic and interphase cells.

We couldn’t use an siRNA approach to work out the RLIP-Epsin association, because knocking down RLIP76 led to a decrease of Epsin in whole cell extracts, a result that would suggest that a large fraction of Epsin is complexed to and stabilized by its interaction with RLIP (data not shown).

Mitotic Phosphorylation of Epsin Depends on RLIP
cdk1 phosphorylates Epsin that is found in a complex with RLIP76 that binds cyclinB/p34cdc2. We tested the hypothesis that RLIP76 is a scaffolding protein that brings together the enzyme, cdk1, and its substrate, Epsin. It is noteworthy that phosphorylation by cdk1 of several proteins involved in endocytosis is concomitant with the shut-off of endocytosis during M phase. The observation that a mutant of Epsin mimicking its phosphorylation by cdk1 is no longer able to interfere with endocytotic functions during mitosis.

The “scaffold” hypothesis was tested by investigating whether the C-terminal region of RLIP76 that corresponds to the region of RLIP that interacts with cyclinB (amino acids 497–655 of p-RLIP) behaves as a dominant negative mutant and blocks Epsin phosphorylation by cdk1 in mitosis. We over-expressed transiently the C terminus of RLIP tagged with a myc epitope. We also overexpressed Epsin, because this gives a clearer view of Epsin mobility shift upon its phosphorylation by cdk1. Fig. 7 shows that overexpressed Epsin displayed a characteristic mobility shift in mitotic HeLa cells. Overexpression of these proteins, alone or together, failed to impair the entry of cells in M phase, as witnessed by the increase in cyclinB1 in cell extracts. However, expression of the C terminus of RLIP76 totally inhibited the mitotic shift of Epsin, hence, its phosphorylation by cdk1; the unique band migrated at the same molecular weight as interphasic Epsin, and the amount are around the sum of the two bands seen in mitosis. The alpha subunit of AP2 also displayed a mitotic shift most probably due to its phosphorylation by a kinase different from cdk1 (13); this shift was not affected by expression of the C terminus of RLIP76 (data not shown). To address the question of the specificity of the effect of the C terminus of RLIP76 upon the phosphorylation of Epsin, we tested whole mitotic cell extracts with MPM2, a monoclonal antibody that recognizes mitotic phosphoserines and phosphothreonines (13, 22). Fig. 8A shows that there was no difference between control cells and cells transfected with the C terminus of RLIP76 or the Ral-binding
domain of RLIP76 for all three proteins. Roughly the phospho-pattern in mitotic cells were not modified in conditions where phosphorylation of Epsin is inhibited. Nucleolin is another substrate of cdk1 (23), and the TG3 antibody (24) specifically recognizes nucleolin phosphorylated by cdk1. Fig. 8A shows that neither the C terminus of RLIP76 nor the Ral-binding domain prevented the phosphorylation of nucleolin. CyclinB1 was equivalent in all cell extracts, showing that cells were correctly synchronized. Erk was used as a loading control. Fig. 8B shows that, in mitotic cells, there was no difference in the amount of p34cdc2, detected with an anti-PSTAIRE, whether the cells were expressing or not the C terminus of RLIP76. Finally, cdk1 activity measured by a histone H1 kinase assay displayed no difference related to the expression of full-length RLIP76 or the C terminus of RLIP76 as compared with control cells (Fig. 8C). These results confirm that mitosis proceeds while the C terminus of RLIP76 is overexpressed, that global cdk1 activity is not altered, and that the effect observed on the cdk1 phosphorylation of Epsin is specific.

Because RLIP76 is an effector of the Ral GTPase, we wondered whether Ral signaled to cdk1 via RLIP76. We overexpressed the Ral binding domain of RLIP that blocks Ral signaling (6, 25) and investigated its effect on the phosphorylation of Epsin in mitotic cells. Fig. 7 shows that, although blocking Ral signaling did not impair entry in mitosis (cyclinB is normally increased), it indeed inhibited Epsin phosphorylation, while expression of full-length RLIP76 had no effect (Fig. 7B). A similar effect was obtained by expressing another Ral binding domain, from Sec5 (6) (data not shown).
DISCUSSION

Several interphase processes are stopped when cells undergo mitosis, and these resume late in mitosis or at the next G1 cycle. Such is the case for endocytosis, although it is not clear whether endocytosis must stop for mitosis to proceed or whether the absence of endocytosis is a physical consequence of the mitotic process. In cells undergoing mitosis, coated pit invagination is inhibited \textit{in vitro} by cdk1 (10) and several molecules involved in endocytosis are phosphorylated by the M-phase cdk1 kinase. This phosphorylation is supposed to modify substrate subcellular localization (26, 27) and/or protein-protein affinity (13).

During interphase, RLIP76/RalBP1 interacts with POB1 and AP2 (8, 18, 28) and is found in a complex with Epsin and Numb. It plays a role in the endocytosis of transmembrane receptors (8, 9). At the onset of mitosis, cdk1 phosphorylates POB1 and Epsin, with no apparent consequence for the former. Phosphorylated Epsin was shown to lose its capacity to bind POB1 and AP2 (13, 21). RLIP76 itself is not a target of cdk1 (13).

RLIP76/RalBP1 plays a pivotal role by providing a link between the Ras signaling machinery that transduces extracellular signals and the machinery involved in endocytosis. Signal transduction from Ras to RLIP proceeds via RalGEFs and the Ral GTPases. This Ral signaling module was shown to be involved in receptor endocytosis (8, 9). It is noteworthy that all of the protein-protein interactions converging to or diverging from RLIP are conserved between flies and humans (12). In mitotic cells, RLIP76 is found at the centrosome.

FIG. 7. RLIP76 is required for Epsin phosphorylation at mitosis. \textbf{A}, HeLa cells were transfected with a plasmid expressing Epsin and a plasmid expressing the indicated proteins tagged with a Myc epitope. \textit{RLIP} C-ter, the last 159 amino acids of RLIP76; \textit{RalBD}, the Ral binding domain of RLIP76 (6). Whole cell extracts were immunoblotted for Epsin, cyclinB1, and Myc epitope. \textbf{B}, same experiment as in \textbf{A} with full-length RLIP76 and the C terminus of RLIP76, tagged with Myc epitope.

FIG. 8. RLIP76 is not required for cdk1 activity. \textbf{A}, HeLa cells were transfected with a plasmid expressing the C terminus of RLIP76 or the Ral binding of Sec5. Cell extracts from mitotic cells (M) and from exponentially growing cells (E) were immunoblotted and tested for mitotic phosphorylation (MPM2), cyclinB1, phosphorylation of nucleolin, and Erk1/2 as a loading control. \textbf{B}, same experiment as in \textbf{A} with full-length RLIP76 and the C terminus of RLIP76, tagged with Myc epitope. \textbf{C}, cell extracts from mitotic cells (M) expressing RLIP76 or its C terminus were incubated with beads loaded with p13. After extensive washing, beads were incubated with histone H1 (3 \(\mu\)g) and [\(\gamma\)-\(^32\)P]ATP, and histone H1 phosphorylation was detected by autoradiography after SDS-PAGE.
where it is required for separation of the duplicated centromeres (11).

In a search for partners of RLIP76 and more specifically for partners involved in RLIP function during mitosis, a yeast two-hybrid screen and subsequent two-hybrid assays revealed that cyclinB protein interacts with both fly and human RLIP proteins. These interactions were confirmed in vivo with denaturing gels. We also show that RLIP76 associates with a catalytically active cyclinBp34cdc2/cdk1 complex. This is consistent with the fact that in cells almost all cyclinB1 is complexed with p34cdc2 whereas some free inactive p34cdc2 kinase is present.

To shed light on the functional relationship between endocytic complexes and the interaction between RLIP76 and cdk1, we tested the cell cycle dependence of the association between RLIP76 and its partners involved in trafficking. We found that RLIP76 can be immunoprecipitated together with Epsin, Numb, and AP2 during interphase as well as during mitosis. Reciprocally, Epsin can be precipitated together with AP2 independently of the phase of the cell cycle. These data differ from those published in two other studies: (i) in brain extracts treated with Xenopus mitotic extracts, Epsin loses affinity for AP2 (21); (ii) pull-down experiments with CHO cells show that overexpressed Epsin loses its affinity for the alpha subunit of AP2, during mitosis and that the AP2-Epsin interaction displays a lower stability in mitosis versus in interphase (13). We don’t know the reasons for this difference, except that the cell types are not the same. We also show that the C-terminal region of RLIP76, equivalent to the region of b-RIP that interacts with cyclinB and POB1, behaves as a dominant negative mutant that inhibits the mitotic phosphorylation of Epsin. These results suggest the existence of a connection that brings together cdk1 and its substrate Epsin via their interactions with RLIP76.

We questioned whether the Ral-RLIP76 signaling cascade was involved in this mitotic function of RLIP76. Because both a dominant active (Ral G23V) as well as a dominant negative (Ral S25N) allele of Ral led to cell death upon nocodazole treatment (data not shown), we blocked Ral signaling by over-expressing the Ral binding domain of RLIP76 (Fig. 7) or Sec5 (not shown). Both situations led to a block of Epsin phosphorylation, although cell cycle progression reflected by the increase in cyclinB1 level in G2/M was normal, as was the amount of p34cdc2 as well as cdk1 activity, measured by a histone H1 kinase assay, and reflected by nucleolin phosphorylation and the global mitotic phospho-pattern. The fact that the same effect was observed by expressing either of these Ral binding domains strongly argues that Epsin phosphorylation requires Ral signaling.

We propose that RLIP76/RalBP1 serves as a scaffold protein that brings together proteins forming an endocytotic complex during interphase and cdk1 to switch off endocytosis. One of its substrates would be Epsin; phosphorylated Epsin has lost its capacity to be competent for endocytosis as suggested by its inability to block endocytosis when overexpressed, as opposed to unphosphorylated Epsin (13). Whether the role of RLIP76 as a scaffold for the phosphorylation of Epsin by cyclinBp34cdc2 could be extended to some other proteins binding directly or indirectly RLIP is under investigation.

The capacity of RLIP76 to facilitate phosphorylation of Epsin by cdk1 appears dependent on Ral signaling. This might be due to a destabilization of the RLIP-Epsin complex, which would also point to the matchmaking function of RLIP, in order for Epsin to become phosphorylated by cdk1. Alternatively, it might be due to a stabilizing effect of Ral-GTP, once bound to RLIP, on the cdk1-RLIP76 complex. This would point to the requirement of some Ral signaling during G2/M to switch off endocytosis, in contrast to the proposed requirement of Ral activation for the endocytosis of activated receptors during interphase.

Finally, we have shown that RLIP is also complexed with Numb, a protein involved together with p34cdc2 in the asymmetric division of fly neuroblasts (31), a process where AP2 was recently also found involved (6). It is noteworthy that, despite the presence of a conserved consensus cdk1 phosphorylation site in both fly and human Numb, we failed to phosphorylate immunoprecipitated human Numb with a mitotic cell extract pulled-down with p13 (data not shown). However, the complex formed by RLIP, cdk1, Numb, and AP2 might be the place where these proteins meet to play their parts in asymmetric division.

Is there any other role for an association between RLIP and cdk1? RLIP76 is also found associated with spindle microtubules (11), and a target for cdk1 bound to RLIP could be the microtubule network where MAP2 and MAP4 are phosphorylated by cdk1 (29, 30) with a subsequent inhibition of their microtubule-stabilizing and -nucleating activity. It has not escaped our notice that this molecular function of RLIP proteins and of Ral might also be used during mitosis. RLIP and cdk1 are both present on mitotic centrosomes where RLIP might bring together cdk1 and substrates that require phosphorylation to be active during spindle pole separation, a process where both cdk1 and RLIP76 are implicated.

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RLIP, an Effector of the Ral GTPases, Is a Platform for Cdk1 to Phosphorylate Epsin during the Switch Off of Endocytosis in Mitosis

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