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Biochemical and Biophysical Research Communications 334 (2005) 128–133
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The Rab-interacting lysosomal protein, a Rab7 and Rab34 effector, is capable of self-interaction ☆

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Received 13 June 2005
Available online 27 June 2005

Abstract

Rab-interacting lysosomal protein (RILP) has been identified as an interacting partner of the small GTPases Rab7 and Rab34. Active Rab7 recruits RILP on the late endosomal/lysosomal membrane and RILP then functions as a Rab7 effector controlling transport to degradative compartments. Indeed, RILP induces recruitment of dynein–dynactin motor complexes to Rab7-containing late endosomes and lysosomes. Recently, Rab7 and RILP have been found to be key proteins also for the biogenesis of phagolysosomes. Therefore, RILP represents probably an important factor for all endocytic routes to lysosomes. In this study, we show, using the yeast two-hybrid system, that RILP is able to interact with itself. The data obtained with the two-hybrid system were confirmed using co-immunoprecipitation in HeLa cells. The data together indicate that RILP, as already demonstrated for several other Rab effector proteins, is capable of self-association, thus probably forming a homo-dimer.

Keywords: RILP; Rab7; Rab34; Membrane traffic; Lysosome; Late endosome; Endocytosis; Oligomerization; Two-hybrid

Rab-interacting lysosomal protein (RILP) is a common effector of Rab7 and Rab34, two Rab proteins implicated in the biogenesis of lysosomes [1–4]. Rab GTPases and their effectors are considered key regulatory factors for membrane traffic [5–10]. Indeed, Rab proteins organize distinct protein complexes within a single vesicle/organelle and act to regulate all stages of vesicular transport. In particular, several Rab proteins have been implicated in the movement of vesicles/organelles and/or associated to specific microtubules- or actin-based motor complexes [10]. Rab7, in its active GTP-bound form, is able to recruit RILP, which, in turn, is able to recruit, on Rab7-positive organelles, the functional dynein–dynactin motor complexes [1,2]. These organelles are then transported by these motors toward the minus end of microtubules and therefore are cleared from cell periphery [1,2,4]. Consequently, Rab7 and RILP are involved in the regulation of late endocytic transport steps, lysosomal distribution, and lysosomal morphology. Indeed, expression in HeLa cells of Rab7 dominant negative mutants or of a truncated mutant of RILP containing the Rab7, but not the dynein–dynactin motor binding domain, strongly inhibits transport of endocytic markers to lysosomes [1,2,11–13]. Rab7 and RILP have been involved also in the phagocytic process [14–17]. Indeed, it has been demonstrated that full maturation of phagosomes to phagolysosomes requires the retrograde emission of tubular extension which are generated by activation of Rab7, recruitment of RILP, and, in turn, recruitment of the dynein–dynactin microtubule associated motor complexes [14,15]. Rab proteins and their effectors play also

☆ *Abbreviations:* RILP, Rab-interacting lysosomal protein; PNS, post-nuclear supernatant; GST, glutathione-*S*-transferase; EGFP, enhanced green fluorescent protein.

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doi:10.1016/j.bbrc.2005.06.067

a key role in the maturation of parasite-containing organelles, and, in particular, Rab7 and RILP seem to be crucial for the maturation of *Salmonella* invasion vacuoles [18–25]. In addition, there is recent evidence that Rab7 is essential for the final maturation of late autophagic vacuoles [26,27] although the role of RILP in this process has not yet been investigated.

Several Rab and Rab effectors have been shown to dimerize, and dimerization profoundly affect function of these proteins since disruption of the ability to self-interact causes loss of function [28–34].

In this study, we performed a two-hybrid screening using RILP as bait. Interestingly, some positive clones were RILP itself indicating that RILP could probably dimerize in the yeast two-hybrid system. We then characterize RILP with respect to its ability to self-interact. RILP has two coiled-coil domains [1]. Coiled-coil proteins are involved in a variety of organizational and regulatory processes in eukaryotic cells. They provide cables and networks in the cyto- and nucleo-skeleton, and molecular scaffolds that organize membrane systems. Indeed, several coiled-coil proteins are involved in regulation of membrane traffic [35–39]. Here, we demonstrate, using the two-hybrid technology and co-immunoprecipitation, that RILP is able to self-interact. Indeed, RILP is probably present in the cytosol as a dimer and dimerization depends upon the presence of its coiled-coil predicted sequences. On the basis of this result, we discuss the role of oligomerization of Rab and Rab effector proteins in membrane traffic.

Materials and methods

Reagents. Restriction and modification enzymes were from Boehringer or Biolabs while chemicals were from Sigma–Aldrich.

cDNA cloning and plasmid construction. pGADGH-RILP-C33 has been isolated previously in a two-hybrid screen using Rab7Q67L as bait [1,40]. Full-length human RILP (RILP) was amplified by PCR using 5′ and 3′ primers containing the *Eco*RI site. The fragment was then subcloned in the *Eco*RI site of pGBKT7, pLexA or pCEFL-HA to yield Gal4BD-RILP, LexABD-RILP, and HA-RILP, respectively. pEGFP-RILP-C33 was obtained by inserting in pEGFPC1 an *Eco*RI–*Sal*I fragment encoding the C-terminal half of RILP (RILP-C33; [1]). pGBKT7-RILP-C33 was obtained by inserting an *Eco*RI–*Not*I fragment encoding the C-terminal half of RILP (RILP-C33; [1]). pEGFP-RILP was obtained by inserting in pEGFPC1 cut with *Sal*I–*Bgl*II a *Sal*I/*Bam*HI (partial digestion) fragment encoding full-length RILP. The C-terminal deletions of RILP in the pGBKT7 or pLexA vectors were made using the following restriction enzymes: *Bam*HI to obtain RILPΔC1, *Sma*I to obtain RILPΔC2, and *Nae*I to obtain RILPΔC3; these three constructs encode three hybrid proteins containing the Gal4 or LexA binding domain and 321, 201 or 84 aa of RILP, respectively. The RILPΔN construct contains an internal *Sma*I deletion that eliminates the amino acids from position 8–202.

Antibodies. The rabbit polyclonal antibody against GFP was from Clontech while the mouse monoclonal antibody against the HA epitope was from Sigma (H 6908). Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Sigma. Unless indicated otherwise the dilution of antibodies was 1:500.

Two-hybrid method. The pGBKT7-RILP construct was used to screen a liver cDNA library made in the pACT2 vector obtained from Clontech using the *Saccharomyces cerevisiae* AH109 reporter strain [41–44]. The transformants were plated on synthetic medium lacking leucine and tryptophan. After 2 days of growth at 30 °C, colonies were picked and assayed for growth at 30 °C on synthetic medium lacking histidine, leucine, adenine, and tryptophan, and containing 5 mM 3-amino-1,2,4-triazole (a competitive inhibitor of the His3 protein) after 3 days of growth at 30 °C, His^+ and Ade^+ colonies were transferred onto nitrocellulose filters and assayed for β-galactosidase activity, using 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside as a substrate. His^+, Ade^+, and $LacZ^+$ colonies were indicative of positive interaction. A total of 120 transformants were His^+ Ade^+ $LacZ^+$. Plasmid DNAs from these transformants were obtained and used to transform HB101 *Escherichia coli* cells. The recovered library plasmids were then tested for interaction with Lamin C, Ras or RILP; 40 clones transactivated the reporters only in the presence of the ''bait'' (pGBKT7-RILP). Three of the clones encoded RILP. To test interaction between RILP and RILP deletion mutants, pLexA or pGBKT7 and pGadGH or pACT2 vectors containing the appropriate insert were co-transformed into *S. cerevisiae* L40 or AH109 reporter strains [41–44]. Yeast colonies were then assayed for growth on synthetic medium lacking histidine, leucine, adenine, and tryptophan, and for β-galactosidase activity. To quantify the interactions, we performed a liquid β-galactosidase assay using *o*-nitrophenyl-β-D-galactoside as a substrate [43,45].

Cell culture and transfection. Tissue culture reagents were from Gibco. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, 100 U/ml penicillin, and 10 μg/ml streptomycin, in a 5% CO_2 incubator at 37 °C. Transfection was performed using the DOTAP or DOSPER transfection reagent from Boehringer–Mannheim as previously described [12]. Cells were transfected with pEGFP-RILP, pEGFP-RILP-C33, and/or PCEFL-HA-RILP for 18–24 h and then processed for biochemical assays.

Immunoprecipitation. HeLa cells were co-transfected with plasmids encoding HA-RILP and GFP-RILP or GFP-RILP-C33 as indicated. After transfections, cells were lysed in 0.2% NP40 or Triton X-100, 10 mM Tris–HCl, pH 7.5, 150 mM NaCl, and 2 mM EDTA (buffer A). Post-nuclear supernatants (PNS) were incubated for 2 h at 4 °C with the appropriate antibody, then protein G–Sepharose or protein A–Sepharose was added, and samples were further incubated for 2 h at 4 °C. Beads were then washed three times with buffer A, once with buffer B (10 mM Tris–HCl, pH 7.5), resuspended in Laemmli buffer, and loaded onto SDS–PAGE.

Western blot. Separated proteins on SDS–PAGE were transferred onto nitrocellulose membrane, and then probed with the appropriate primary and secondary antibodies. The bands were visualized using the enhanced chemiluminescence system (ECL; Amersham).

Results and discussion

RILP is able to self-interact in the two-hybrid system

A yeast two-hybrid screen was used to isolate proteins that interact with RILP. A Gal4BD-RILP fusion construct was made in pGBKT7 vector and used in the screen to isolate putative interactors. Expression of the fusion protein was confirmed by Western blot analysis of total yeast extracts with and anti-RILP antiserum (data not shown). The construct was used to screen a liver cDNA library encoding proteins as C-terminal fusions with the transcriptional activation domain of Gal4

in the pACT2 vector. From 2×10^6 primary transformants 120 were His^+LacZ^+ and only 40 transformants were encoding true positives that did not activate transcription in the presence of a non-specific test bait. Three transformants encoded RILP itself suggesting that RILP was able to self-interact. The structural analysis of RILP primary amino acid sequence by computer-assisted predictions reveals that its N- and C-terminal regions contain heptad repeats characteristic of coiled-coil domains [1]. Coiled-coil domains consist of seven amino acid repeats where position one and four are predominantly non-polar, and position five and seven are mainly polar. Their overall structure is α-helical. Two regions display the highest probability of forming coiled-coil structures on RILP: the first one between amino acids 75 and 180, and the second one between amino acids 245 and 280. Coiled-coil domains are often responsible for protein–protein interaction and, in particular, for self-interaction. Therefore, we decided to further investigate using the two-hybrid technology whether and how RILP was able to self-interact. To be able to test the RILP self-interaction, we constructed the pGADGH-RILP plasmid as described under Materials and methods. Fig. 1 shows the results of the two-hybrid test. Lamin C was used as negative control because it contains a coiled-coil domain, while interaction between pLexA-Ras and pGAD-Raf was used as a positive control [46,47]. The interaction was revealed by the growth of yeast cells on His-synthetic medium. As expected, Rab7 interacted with RILP and RILP-C33 but did not interact with Lamin C or Raf, while Ras was able to interact only with Raf. RILP was clearly able to self-interact and to interact with its truncated form RILP-C33, while no interaction was detected with Lamin C or Raf.

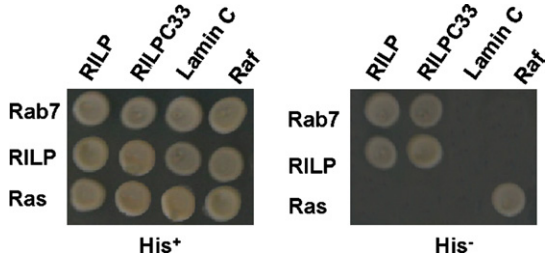

Fig. 1. RILP is able to self-interact in the yeast two-hybrid system. *HIS3* reporter gene activation caused by specific interaction between various bait and prey RILP fusions in the two-hybrid system. L40 reporter yeast cells co-transformed with pGADGH plasmid and with pLexA plasmid expressing the indicated proteins were spotted on synthetic complete medium lacking tryptophan and leucine (His^+) or lacking tryptophan, leucine, and histidine (His^-). RILP self-interaction and interaction between the full-length molecule and the truncated RILP-C33 sequence encoding C-terminal 185 aa of the protein was detected. Lamin C was used as a control of non-specific interactions while the interaction between Ras and Raf was monitored as a positive control.

These results were confirmed using the other reporter gene, β-galactosidase. Indeed, β-galactosidase activity, as judged by a colorimetric assay described under Materials and methods, was present in all the yeast colonies able to grow on synthetic medium lacking histidine, leucine, and tryptophan (data not shown).

These results demonstrate that, in the two-hybrid assay, RILP is able to self-interact.

RILP is able to self-interact in HeLa cells

To confirm the self-association of RILP in vivo, HeLa cells were transfected with GFP-RILP and/or HA-RILP. After transfection the cells were lysed and subjected to immunoprecipitation analysis with anti-GFP or anti-HA antibodies. Subsequently, immunoprecipitates were loaded onto SDS–PAGE and subjected to Western blot analysis using anti-HA or anti-GFP antibodies. The results of these experiments are shown in Fig. 2. Fig. 2A shows control lanes where immunoprecipitates from cells expressing HA-RILP were not recognized by anti-GFP antibodies and immunoprecipitates from cells expressing GFP-RILP were not recognized by anti-HA antibodies. In co-transfected cells (Fig. 2B), anti-HA and anti-GFP were able to recognize the corresponding immunoprecipitates but also anti-HA detected HA-RILP in immunoprecipitates made with anti-GFP.

These data demonstrate that RILP in vivo is in a complex with more than one molecule of RILP, probably self-interacting. If HeLa cells were co-transfected with HA-RILP and GFP-RILP-C33, again anti-HA antibodies were able to recognize a band corresponding to HA-RILP in immunoprecipitates obtained with anti-GFP antibodies, demonstrating that the C-terminal half of the protein (RILP-C33) is capable of associating with full-length RILP and strongly indicating that the C-terminal part of the protein is involved in the interaction.

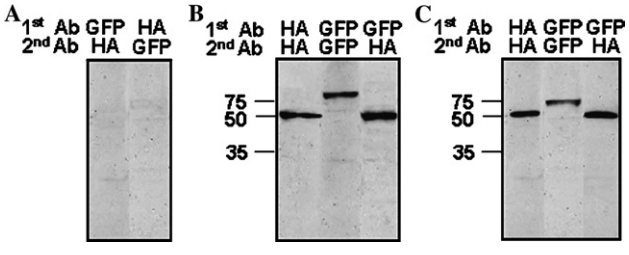

Fig. 2. RILP is able to self-interact in vivo. HeLa cells were transfected with plasmids encoding HA-RILP or GFP-RILP (A), co-transfected with plasmids encoding HA-RILP and GFP-RILP (B) or GFP-RILP-C33 (C) as indicated. After 18 h cells were detergent extracted and the post-nuclear supernatants (PNS) were subjected to immunoprecipitation with the first antibody as indicated. The immunoprecipitates were loaded onto SDS–PAGE, transferred to a nitrocellulose filter, and subjected to Western blot analysis using the second antibody as indicated.

These data demonstrate that the two epitope-tagged RILP proteins interact in vivo (Fig. 2C). The co-immunoprecipitation was specific, as anti-HA antibodies were not able to immunoprecipitate GFP-RILP and anti-GFP antibodies were not able to immunoprecipitate HA-RILP.

Moreover, purified recombinant GST-tagged RILP from *E. coli* was able to pull down RILP from extracts of HeLa cells and/or from a solution of purified RILP (data not shown).

Mapping the domains involved in the interaction

To further map the domains involved in the RILP self-interaction, we made three C-terminal deletion constructs of RILP, RILPΔC1, RILPΔC2, and RILPΔC3 in the two-hybrid vectors (Fig. 3A). RILPΔC1, RILPΔC2, and RILPΔC3 code for 323, 201, and 84 aa of RILP, respectively. Therefore, while RILPΔC1 contains both the coiled-coil domains, RILPΔC2 has only the first one while RILPΔC3 contains only a very short part of the first coiled-coil domain. In addition, the construct RILPΔN contains only the second (245–280 aa) coiled-coil domain. A scheme depicting all these constructs is shown in Fig. 3A. The results of the two-hybrid assay set up to measure interaction between the proteins encoded by the deletion constructs and full-length RILP or RILP-C33 are shown in Fig. 3B. Rab7 and Ras were used as positive and negative control, respectively. As expected, full-length RILP and RILP-C33 were interacting strongly with Rab7 but were not interacting at all with the Ras GTPase (Fig. 3B). Full-length RILP was able to interact very strongly with itself but also with N-terminal and C-terminal deletion mutants (Fig. 3B). RILP-C33 was able to interact with itself and with the RILPΔN but, surprisingly, also with the three C-terminal deletion mutants, although to a different extent, as quantified by measuring relative units of β-galactosidase activity (Fig. 3B).

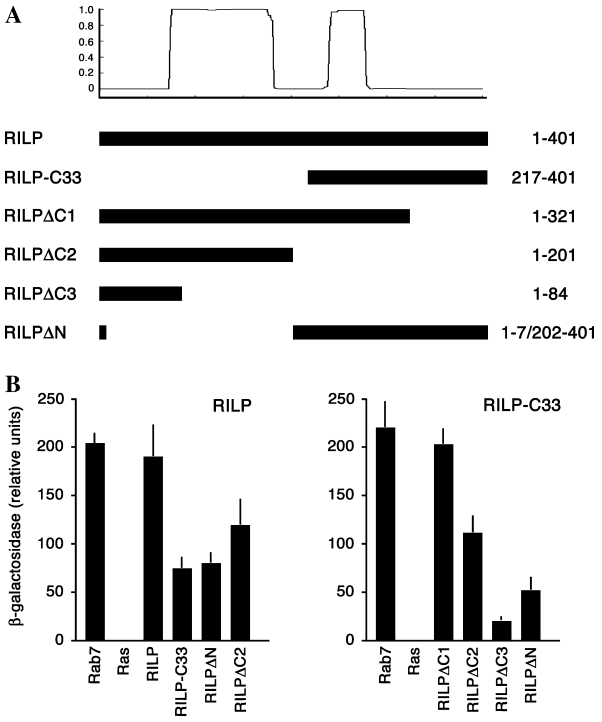

Fig. 3. Analysis of the self-interaction of RILP using deletion mutants. (A) Scheme depicting the different deletion mutants used in this study. On top of the scheme a histogram indicating the probability of forming α-helical coiled-coil as determined by Lupas algorithm with a window of 28, where the *y*-axis indicates the probability (0–1). (B) Quantification of the β-galactosidase activity. Liquid β-galactosidase assay was performed as described under Materials and methods. The values are means of four independent experiments made in duplicate ± the standard error.

Some Rab and Rab interacting proteins have been shown to dimerize, and oligomerization is one of the fundamental features for their physiological function [28,30–32,34,48–50]. Indeed, it has been shown that Rabaptin5 and EEA1, two Rab5 effectors, are present in a homo-dimeric state, and dimerization depends upon the presence of its coiled-coil predicted sequences [34,48,50]. ALS2, a GEF (guanine nucleotide exchange factor) for Rab5, forms a homophilic oligomer through its C-terminal regions; this homo-oligomerization is crucial for the Rab5GEF activity in vitro and the ALS2-mediated endosome enlargement in the cells [31]. The novel Rab11-FIP/Rip/RCP family of proteins displays extensive homo- and hetero-interacting abilities, and these interactions primarily involve their C-terminal ends [29]. The small GTPases Rab5, Rab9, and Rab11, essential regulators of vesicular transport, form dimers that could represent an inactive, membrane-bound pool of these Rab proteins [28,32,49]. Dimerization of these Rab proteins depends on the nucleotide-bound conformation, is independent of lipid modification and could allow these proteins to undergo GDP/GTP cycles without recycling to the cytosol [28,32,49].

Therefore, dimerization seems to be a common feature of several Rab and Rab effector proteins. Our data indicate that RILP (the Rab7 and Rab 34 effector) is able to interact with itself, thus probably forming dimers. While this paper was in preparation, the crystal structure of Rab7-GTP in complex with the Rab7 binding domain of RILP was determined [51]. Interestingly, the structure shows that the Rab7 binding domain of RILP forms a homo-dimer with two surfaces for binding of two Rab7 molecules. As suggested by the authors, this finding prompts to speculate that Rab7 and RILP could influence each other regarding membrane targeting [51]. Our results confirm the structural data although indicating also a possibly more complex situation. Indeed, from the

two-hybrid analysis the C-terminal part of the protein seems to interact with itself but also (although to lesser extent) to the N-terminal part of the protein containing the first (and stronger) coiled-coil domain. Moreover, full-length RILP seems to interact equally well with the C-terminal and N-terminal deletion proteins suggesting that its dimerization is probably not confined to the C-terminal region. Structural studies involving the entire RILP protein will be requested to map all the domains involved in RILP dimerization. Moreover, future work is needed to show the functional meaning of this dimerization in terms of membrane targeting and control of transport to late endocytic compartment.

Acknowledgments

This work was partially supported by grants from Ministero dell'Università e della Ricerca Scientifica to Cecilia Bucci (60%, Progetti di Ricerca di Interesse Nazionale 2002, 2004). We thank Pietro Alifano for critical reading of the manuscript.

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