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Dimerization Properties of Rabaptin-5 and Its Isoforms

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Abstract—Rabaptin-5 plays an important role in intracellular membrane traffic acting as an effector molecule of small GTPases Rab5 and Rab4. It was previously demonstrated that Rabaptin-5 exists as a part of a large protein complex *in vivo* and is able to form dimers *in vitro*. Data of X-ray structural analysis suggest that dimerization of Rabaptin-5 is an important feature required for its interaction with Rab5 GTPase. Recently several isoforms of Rabaptin-5 characterized by various deletions in the polypeptide chains have been identified. These isoforms might exhibit functional properties that differ from those of Rabaptin-5. In this study, we have investigated dimerization properties of δ and γ isoforms of Rabaptin-5. In addition, we have provided the first direct evidence for Rabaptin-5 dimerization in cells.

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Key words: Rabaptin-5, Rabaptin-5 isoforms, dimerization, membrane transport

Rab family GTPases play a key role in the regulation of various intracellular membrane traffic pathways, and their functions are realized through interactions with effector molecules. There are more than 40 various Rab GTPases, each of which regulates specific steps of membrane transport [1]. The same GTPase may be involved in regulation of various processes by interacting with different effector molecules. GTPases Rab5 and Rab4 are regulators of early endosome transport and recycling of early endosomes, respectively [2, 3]. Determining sequential membrane domains in vesicular transport [4], Rab5 and Rab4 may recruit the same effector molecules, which probably underlie "succession" of Rab5- and Rab4-positive membrane domains and direction of membrane transport from Rab5-positive to Rab4-positive domain [5, 6]. Rabaptin-5 is one such effector of GTPases Rab5 and Rab4. This protein was originally identified as a Rab5 effector in early endosome fusion. Rabaptin-5 exists in a cell as a component of a large multiprotein complex and is recruited to membrane by the GTP-bound form of Rab5 (Rab5-GTP) [7]. The role of Rabaptin-5 as a Rab5 effector in early endosome fusion is currently considered to bring a Rab5 GDP/GTP exchange factor, Rabex-5, which is complexed with Rabaptin-5, thus increasing local concentration of Rab5-GTP [8, 9]. Some experimental evidence exists that Rabaptin-5 forms homodimers [6, 8]. Rabaptin-5 may also sequentially interact with GTPase Rab4 via Rab4-binding domain located at the N-terminus of the protein [6] and directly influence Rab4-regulated process of early endosome return to cell surface [10]. There are data suggesting involvement of Rabaptin-5 in the transport process from *trans*-Golgi network to cell surface [11, 12]. Recently, it has been found that *rabaptin-5* transcript can undergo alternative splicing, which results in appearance of similar proteins, denominated as Rabaptin-5 isoforms, which bear various deletions in the polypeptide chain [13-15]. These deletions may influence functioning of these isoforms as Rab GTPase effector molecules. Deletions in the Rabaptin-5 isoforms may influence the composition of the multiprotein complexes containing Rabaptin-5 isoforms instead of Rabaptin-5. This may result in appearance of new or impairments of preexisting sites for interaction between components of this complex or cause such impairments by alterations of spatial structure of this protein. Indeed, earlier we have demonstrated that δ isoform of Rabaptin-5 exhibits a different spectrum of protein interactions than Rabaptin-5, and this may result in its functional differences from Rabaptin-5 [16]. Interestingly, deletions in the Rabaptin-5 isoforms are located within regions of

Abbreviations: GAL4BD) DNA-binding domain of the transcription factor GAL4; GAL4AD) activation domain of the transcription factor GAL4; Rab5-GTP) GTP-bound form of Rab5.

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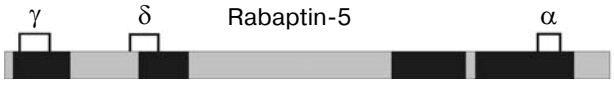

Fig. 1. Scheme of Rabaptin-5 protein structure. Black color shows coiled-coil regions responsible for Rabaptin-5 dimerization. Fragments of the polypeptide chain lacking in γ, δ, and α isoforms of Rabaptin-5 are also shown.

coiled coil of N- (δ and γ isoform) and C- (α isoform) terminal domains, which are responsible for dimerization of Rabaptin-5 molecules (Fig. 1). Taking into consideration the importance of Rabaptin-5 dimerization for its interaction with Rab5 [17], it is essential to elucidate whether Rabaptin-5 isoforms with impaired coiled-coil regions can form dimers. It is also interesting to evaluate the possibility of formation of heteromeric complexes between Rabaptin-5 and its isoforms, which may exhibit different properties due to different properties of Rabaptin-5 and its isoforms.

MATERIALS AND METHODS

Plasmids. Plasmids for analysis of protein–protein interactions, pPC97-Rabaptin-5 and pPC97-Rabaptin-5δ for expression of Rabaptin-5 and Rabaptin-5δ as chimeras with DNA-binding domain of the transcription factor GAL4 (GAL4BD) in yeasts, and also plasmids pPC86-Rabaptin-5 and pPC86-Rabaptin-5δ for expression of corresponding proteins as chimeras with activation domain of the transcription factor GAL4 (GAL4AD), were described earlier [16]. Plasmids for expression of Rabaptin-5γ protein in yeasts (pPC97-Rabaptin-5γ and pPC86-Rabaptin-5γ) were obtained by the same approach [16] using Rabaptin-5γ cDNA and the vectors pPC97 and pPC86 [18]. The plasmid pBK-myc-Rabaptin-5 for expression of the full-size Rabaptin-5 protein with N-terminal *myc*-epitope was obtained by sub-cloning of the coding region of Rabaptin-5 cDNA from the plasmid pPC97-Rabaptin-5 by sites *Sal*I-*Not*I into the vector pBK-myc, responsible for expression of proteins bearing N-terminal *myc*-epitope under control of a cytomegalovirus promoter [16]. The plasmid pRabaptin-5-FLAG for expression of Rabaptin-5 protein with C-terminal FLAG-epitope under control of the cytomegalovirus promoter was obtained by sub-cloning of the fragment of corresponding cDNA by sites *Sal*I and *Not*I from the pGEX-Rabaptin-5-FLAG plasmid between sites *Sal*I and *Not*I in the plasmid pEGFP-N1 (Clontech, USA). The plasmid pGEX-Rabaptin-5-FLAG was obtained on the basis of the vector pGEX-4T-2 (Amersham Biosciences, UK) engineered to contain the sequence coding FLAG-epitope between *Xba*I and *Not*I sites (vector pGEX-C-FLAG) by cloning of Rabaptin-5 cDNA fragment containing AUG codon with inserted *Sal*I site at 5′-end and site *Xba*I instead of termination translation codon at 3′-end of cDNA. The plasmids pRabaptin-5δ-FLAG and pRabaptin-5γ-FLAG for expression of Rabaptin-5δ and Rabaptin-5γ proteins with C-terminal FLAG-epitopes were obtained by similar methods.

Two-hybrid system in yeasts. Protein interaction in yeasts was analyzed using yeast strain Y153 and plasmids based on the vectors pPC97 and pPC86 [18, 19]. Protein interaction was detected using quantitative analysis of the *LacZ* reporter gene activation [20].

Cell lines and transfections. HEK293 cell line was used for expression of proteins. Cells were cultivated in DMEM medium (Gibco, USA) with 10% fetal calf serum (Gibco), 100 units/ml penicillin, and 100 μg/ml streptomycin. For transfection, $5 \cdot 10^5$ cells were seeded onto Petri dishes (30 mm) and transfection was carried out using 0.4 μg plasmid DNA and 0.75 μl of Unifectin-56 transfection reagent kindly provided by A. Surovoi (Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Moscow, Russia). Cells were harvested 24 h after transfection.

Co-immunoprecipitation. The transfected cells were washed three times with cold phosphate-buffered saline (PBS), lysed by pipetting into 120 μl of Tris-buffered saline, pH 8.0 (Sigma, USA), containing 1% Nonidet P40 (Fluka, Germany) and Complete protease inhibitor cocktail (Roche, Switzerland). After incubation on ice for 15 min, the sediment was removed by centrifugation at 4500 rpm in a microcentrifuge for 10 min at 4°C. Lysate (20 μl) was mixed with 20 μl of concentrated 2× gel loading buffer, and proteins were denatured by boiling. The remaining part of the lysate was mixed with 20 μl of anti-FLAG-M2 affinity gel (Sigma) in 300 μl of lysis buffer and binding was carried out for 2 h at 4°C with rotation. The affinity matrix was then washed three times with 400 μl of the lysis buffer. Proteins were eluted by adding 35 μl of the SDS-polyacrylamide gel loading buffer followed by boiling for 5 min. Aliquots (10 μl) of eluates and lysates were separated using 7.5% SDS-PAGE and were then transferred onto Hybond-P membrane (Amersham Biosciences) using semidry transfer. Western-blot analysis was carried out using primary anti-*myc* (clone 9E10) or anti-FLAG-M2 monoclonal antibodies (Sigma; dilution 1 : 2000) and secondary antibodies against mouse IgG conjugated to horseradish peroxidase (Amersham Biosciences; dilution 1 : 5000). The reagent ECL+ (Amersham Biosciences) was used for detection.

RESULTS AND DISCUSSION

Dimerization properties of Rabaptin-5 are determined by two clusters of coiled-coil regions at the N- and C-ends of the polypeptide chain. Each cluster consists of two coiled-coil regions, which interact with identical

sequences of the other molecule thus forming a parallel dimer [6]. Earlier we found two new isoforms of Rabaptin-5 denominated as δ and γ, and characterized by deletions of 40 and 43 amino acid residues in the N-terminal region, respectively [13, 14]. In both these isoforms, the deletions involve the coiled-coil regions responsible for dimerization properties of Rabaptin-5 (Fig. 1). So we have investigated the effects of these deletions on the dimerization properties of δ and γ Rabaptin-5 isoforms. Importance of individual coiled-coil regions of Rabaptin-5 for dimerization was demonstrated in yeast two-hybrid system [6]. This suggests the feasibility of using such an experimental approach for studies of the dimerization properties of Rabaptin-5 isoforms. Studies employed the two-hybrid system in yeasts revealed that in spite of deletions in the regions of the coiled coil, δ and γ isoforms of Rabaptin-5 may form homodimers as effectively as Rabaptin-5 (Fig. 2). Although expression of Rabaptin-5 and its isoforms as chimeras with GAL4BD caused activation of the reporter gene in the absence of putative interaction partners (Fig. 2, GAL4BD-5 + GAL4AD, GAL4BD-5δ + GAL4AD, GAL4BD-5γ + GAL4AD, GAL4BD + GAL4AD-5, GAL4BD + GAL4AD-5δ, GAL4BD + GAL4AD-5γ), co-expression of chimeras of Rabaptin-5 or its isoforms with GAL4BD and GAL4AD resulted in significant additional activation of the reporter gene (Fig. 2, GAL4BD-5 + GAL4AD-5, GAL4BD-5δ + GAL4AD-5δ, GAL4BD-5γ + GAL4AD-5γ). This suggests the existence of homophilic interactions of Rabaptin-5 and its isoforms. Taking into consideration domain organization of Rabaptin-5 in which N- and C-terminal homodimerizing domains are joined by a linker, one can expect that the C-terminal domain of the dimeric isoforms will share structural analogy with C-terminal domain of Rabaptin-5 and will exhibit similar properties, e.g. maintain ability to interact with Rab5. Indeed this suggestion was experimentally confirmed for δ isoform, which (like Rabaptin-5) specifically interacted with Rab5-GTP both *in vitro* and *in vivo* [16]. Further on, we have also analyzed the influence of deletions in δ and γ isoforms on their ability to form heterodimers with Rabaptin-5 and with their counterparts. Results of protein interaction analysis in the yeast two-hybrid system revealed that deletions in the N-terminal domains of these isoforms did not prevent their heteromerization with Rabaptin-5 and with each other (Fig. 2).

The results of analysis of protein interaction in the yeast two-hybrid system underline homo- and heteromerization properties of Rabaptin-5 isoforms and also confirm the ability of Rabaptin-5 to form homomeric complexes. However, analysis of protein interaction in the yeast two-hybrid system may also give false positive results; this seriously complicates the final conclusion on the dimerization properties of Rabaptin-5 isoforms. There is no direct experimental evidence for the existence of intracellular Rabaptin-5 as a dimer. The conclusion on Rabaptin-5 dimerization is based on several facts, which cannot be used for the final conclusion. First, dimerization properties of individual coiled-coil regions were analyzed in the yeast two-hybrid system [6]; such system may

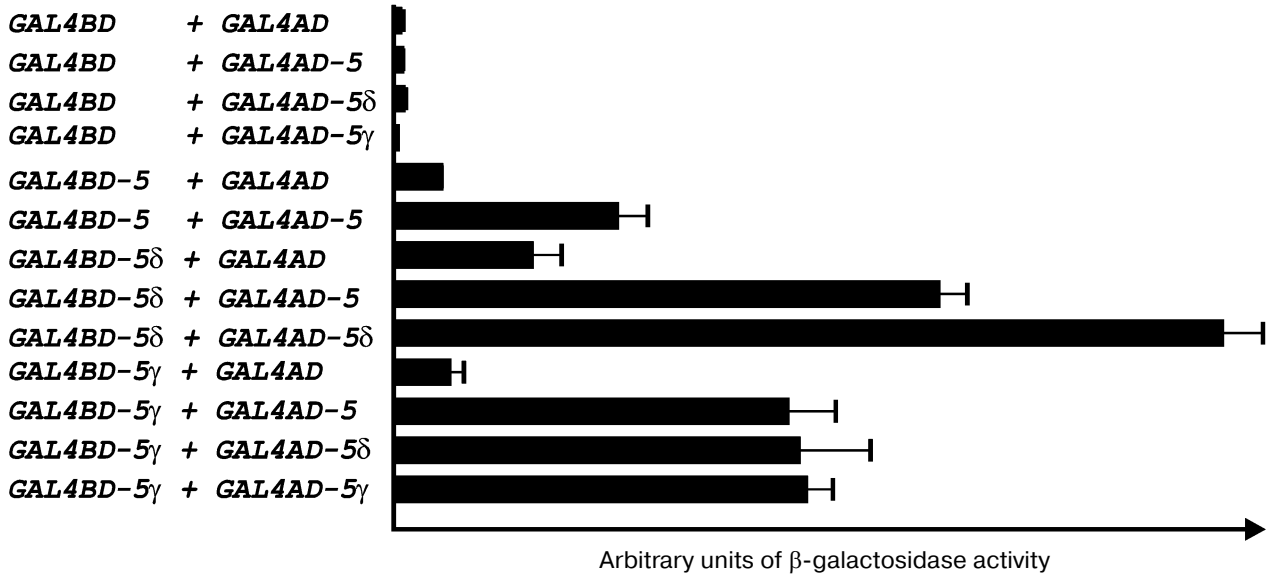

Fig. 2. Analysis of interaction of Rabaptin-5 and its γ and δ isoforms in the yeast two-hybrid system. Yeasts (Y153 strain) were co-transformed with various combinations of plasmids pPC97 (*GAL4BD*), pPC97-Rabaptin-5 (*GAL4BD-5*), pPC97-Rabaptin-5δ (*GAL4BD-5δ*), pPC97-Rabaptin-5γ (*GAL4BD-5γ*) and pPC86 (*GAL4AD*), pPC86-Rabaptin-5 (*GAL4AD-5*), pPC86-Rabaptin-5δ (*GAL4AD-5δ*), pPC86-Rabaptin-5γ (*GAL4AD-5γ*) for expression of GAL4BD or GAL4AD as chimeras of these proteins. Protein interaction was evaluated by activation of the *LacZ* reporter gene. Activities of protein product of the reporter gene *LacZ*, β-galactosidase, are given in arbitrary units. Data represent mean of three independent clones of co-transformants ± SD.

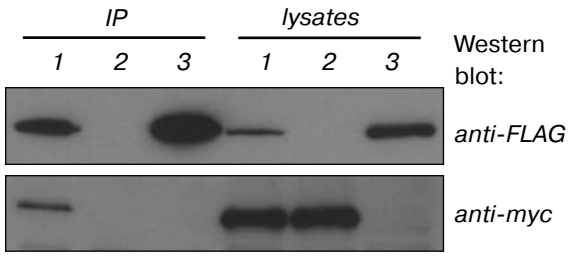

Fig. 3. Rabaptin-5 can form dimers in cells. Western blot analysis of lysates (*lysates*) of HEK293 cells transiently producing *myc*-Rabaptin-5 and Rabaptin-5-FLAG proteins (*1*), *myc*-Rabaptin-5 (*2*) or Rabaptin-5-FLAG (*3*) only. Proteins were immunoprecipitated from corresponding lysates using anti-FLAG affinity gel (*IP*) and Western blotting was done with anti-FLAG-M2 (*anti-FLAG*) or anti-*myc* 9E10 (*anti-myc*) monoclonal antibodies.

yield artifact protein–protein interactions. Second, the presence of Rabaptin-5 *in vivo* as a component of the protein complex was estimated by sedimentation and gel-filtration analyses and also by increased mobility of a protein band exhibiting immunoreactivity with anti-Rabaptin-5 antibodies after cross-linking with cytosolic proteins [6, 8]. However, Rabaptin-5 may interact with some heterologic proteins such as Rabex-5 [8, 9] and γ-adaptin [11], which may represent the actual cause underlying detection of Rabaptin-5 in the large protein complex *in vivo*. Finally, protein cross-linking was accompanied by significant motility of the purified recombinant Rabaptin-5 protein [6]. However, hexahistidine tagged proteins purified from *E. coli* cells under denaturing conditions (such recombinant protein was used in these experiments) often form aggregates, which might be the actual cause of the complex detection. Thus, results available in the literature do not represent exhaustive evidence for Rabaptin-5 dimerization in cells, and other alternative interpretations of the described phenomena are also possible. So it was necessary to analyze in direct experiments the formation of Rabaptin-5 dimers in cells and also to confirm the ability of the δ and γ isoforms to form heterodimeric complexes with Rabaptin-5. We have employed co-immunoprecipitation analysis of Rabaptin-5 proteins with heterologous epitopes from cell lysates. This approach can detect the presence of two molecules of Rabaptin-5 in one complex. Co-expression of Rabaptin-5 with *myc*- and FLAG-epitopes followed by immunoprecipitation of Rabaptin-5 with FLAG-epitope with anti-FLAG antibodies revealed the presence of Rabaptin-5 molecules with *myc*-epitope in the precipitate (Fig. 3). This suggests the presence of at least two molecules of Rabaptin-5 in the protein complex. The size of the protein complex containing Rabaptin-5 was evaluated as 330 kD [8], whereas the size of one molecule of Rabaptin-5 was about 100 kD. Each Rabaptin-5 is in the complex with Rabex-5 molecule of molecular mass of 66 kD [8]. These data together with our results of co-immunoprecipitation analysis indicate that Rabaptin-5 actually exists in cells as homodimer. Similar experiments on co-precipitation of proteins have confirmed data obtained in the yeast two-hybrid system that δ and γ isoforms can form heteromeric complexes with Rabaptin-5 (Fig. 4).

Thus, we have directly demonstrated the existence of Rabaptin-5 as homodimer in cells. It is important to emphasize that dimerization of the C-terminal region of Rabaptin-5 is necessary for its interaction with Rab5 as concluded from results of X-ray analysis, which have demonstrated involvement of amino acid residues of both Rabaptin-5 molecules in a dimer in interaction with Rab5 [17]. Our results also indicate that δ and γ isoforms of Rabaptin-5 can form homo- and heteromeric complexes with each other and also with Rabaptin-5. Based on the model of paired interaction of the individual coiled-coil regions, one can suggest that structures of C-terminal domains of dimers containing Rabaptin-5 isoforms will be identical to those of Rabaptin-5, and so dimers containing Rabaptin-5 isoforms will be able to interact with Rab5. As maintenance of dimerization properties of isoforms is an important precondition for their interaction with Rab5, this suggests that both Rabaptin-5 and its δ

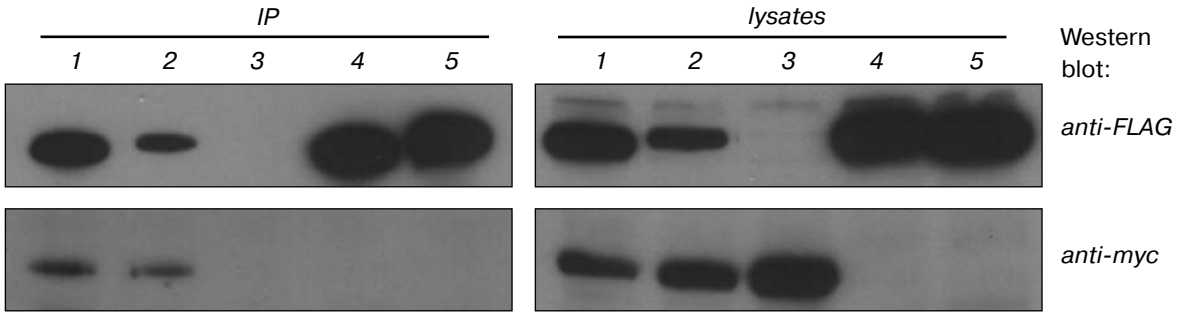

Fig. 4. Rabaptin-5 can form heteromers with its δ and γ isoforms in cells. Western blot analysis of lysates (*lysates*) of HEK293 cells transiently producing *myc*-Rabaptin-5 and Rabaptin-5γ-FLAG (*1*), *myc*-Rabaptin-5 and Rabaptin-5δ-FLAG (*2*), *myc*-Rabaptin-5 (*3*), Rabaptin-5γ-FLAG (*4*) or Rabaptin-5δ-FLAG (*5*) only. Proteins were immunoprecipitated from corresponding lysates using anti-FLAG affinity gel (*IP*) and Western blotting was done with anti-FLAG-M2 (*anti-FLAG*) or anti-*myc* 9E10 (*anti-myc*) monoclonal antibodies.

and γ isoforms may function as effectors of Rab5 during early endosome fusion. For Rabaptin-5δ, this suggestion has been confirmed in experiments on its ability to interact with Rab5 and Rabex-5 [16]. Formation of homo- and heteromeric complexes involving Rabaptin-5 isoforms may also contribute to regulation of membrane traffic pathways. Deletions in Rabaptin-5 isoforms may change the spectrum of molecules potentially interacting with this protein. In its turn, this may modulate properties of the effector complexes as demonstrated for δ isoform of Rabaptin-5 in which deletion causes disruption of the Rab4 binding site [16]. Therefore, formation of different dimers of Rabaptin-5 and its isoforms might increase the repertoire of Rab–effector complexes exhibiting different properties.

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