Scientific report

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Role of tethering factors in the fusion of early endosomes

During the three-year research period of my Postdoctoral Fellowship, I examined the relationship between human endosomal tethering factors. My contributors and me identified new interaction between early endosomal tether Rabenosyn-5 (Rbsn) and Vps18, a subunit of HOPS and CORVET multisubunit tethering complexes. We found that this interaction is dispensable for autophagy, while it is required for endocytic progression. Additionally it also plays role in actin network reorganization and integrin recycling -likely together with Vps45, a Sec1/Munc18 (SM) protein.

We summarized our results in the manuscript titled: Simon-Vecsei Z., Sőth Á., Lőrincz P., Tálas A., Kulcsár PI., Juhász G: Identification of new interactions between endolysosomal tethering factors. The scientific article was published in the Journal of Molecular Biology scientific journal (Impact Factor: 4.76; D1 ranking in Molecular Biology) in 27 March 2021 (Simon-Vecsei et al.).

Human rabenosyn-5 binds to the Class-C protein Vps18

In our earlier study, we identified interaction between Drosophila Rbsn and Vps18 (Lőrincz et al.). Vps18 is a core subunit of HOPS and miniCORVET tethering complexes. We named this latter complex as 'miniCORVET ', since in Drosophila it is composed of four subunits (Vps33, Vps16 , Vps18 and Vps8; instead of six as in yeast). This raises the question, how this truncated complex achieve tethering? One possibility is the interaction with Rbsn, which is another Rab5 effector. This connection was detected in the case of Drosophila proteins. First, we wanted to prove the connection between the human Vps18 and Rbsn proteins with yeast two-hybrid (y2h), coimmunoprecipitaton (coIP) and immunocytochemistry (IC) methods. I amplified the coding sequence of the proteins from HeLa cDNA and cloned into y2h (pGADT7 and pGBKT7) and mammalian expression (with CMV promoters encoding N-terminal HA- or FLAG-tags) vectors. We observed interaction between human Rbsn and Vps18 using y2h system and in anti-FLAG and anti-HA coIP utilizing overexpressed or endogenous proteins as well. Moreover, we found that the proteins localize to the same endosomal structures in HeLa cells.

Rbsn binds to multiple HOPS and CORVET subunits

Since Vps18 is a subunit of both CORVET and HOPS tethering complexes, we investigated, which other subunits of the complexes interact with Rbsn. Interestingly, beside Vps18, we found all of the shared core subunits (Vps16, Vps33 and Vps11) and all of the specific subunits (Vps39, Vps41, Vps8 and Tgfbrap) as a Rbsn interacting partner in coIP experiments. Next, we wanted to know if the observed connections refer to direct or indirect interactions. Since, coIP method detects both types of interactions as well; we turned to y2h method again. We found, that Vps16, Vps33 and Vps39 interact with Rbsn directly. However, these interactions seem to have lower affinity than the binding to Vps18, based on the different growing properties of yeast cells on different selection medium. The interaction between Rbsn and Vps18 can be detected on selection medium lacking adenine (-Ade), while the others showed binding just

when histidine selection was applied. This latter refers to lower protein-protein binding affinity (Paiano et al.).

Identification of the Rbsn-binding site of Vps18

After this, I used truncated forms of the proteins to identify the domains that are required for the implementation of the interaction. I determined the regions of the protein domains according to data from the literature (Van der Kant et al.; Naslavsky et al.), and from protein structure prediction programme Phyre2 (Kelley et al.). Using y2h method I identified the N-terminal part of Rbsn5 (amino acids (aa) 1-421) and the "middle" part of Vps18 (aa 482-854) as the binding regions. Later we narrow the binding site of Vps18 for Rbsn further, and we identified aa 773-854 as binding site. We verified the identified binding region with GST-pull down method as well. For this we cloned, expressed and purified aa 773-854 of Vps18 in E. coli with tandem 6XHistidine- and GST-tags. After affinity purification of the protein, GST pull-down were performed utilizing HEK293 cell lysate. We incubated the GST-agarose-bound aa 773-854 of Vps18 and GST as control with total cells lysate of cells transfected with Rbsn-FLAG. After intensive washing we could detect Rbsn among the pulled down proteins in the case of the aa 773-854 of Vps18, while there was no binding with control GST.
These findings are in line with the literature (Van der Kant et al.), since the RING domain of Vps18 was identified as the Vps16 binding site) which is important for the complex-formation), and beside this Vps18 can bind Rbsn as well.

Beside the experiments above, I aimed to express and purify the Drosophila miniCORVET complex in E.coli and investigate its binding with recombinant Rbsn. Based on my earlier findings, the main challenge is to increase the solubility of the proteins, since the majority of them are directed to inclusion bodies. I used two pET Duet bacterial expression vectors, which codes all of the four subunits of miniCORVET simultaneously, which may increase the solubility of the complex. However, using these type of vectors, the expression level of Vps8 subunit was decreased and the protein could not be detected either with Coomassie Brilliant Blue staining or with western blot (WB) -neither after several adjusting attempts. Based on these, the investigation of the interaction between miniCORVET and Rbsn using full-length recombinant proteins seemed to be too time-consuming and laborious; so I examined the possible function of the identified interaction between human Vps18 and Rbsn.

LC3 lipidation is independent from the Rbsn binding site of Vps18

To this end, first we established a Vps18 KO HEK293 cell line using the CRISPR/Cas9 technique. We generated potential KO cell lines utilizing the method published by Tálas et al. in collaboration with the authors (Institute of Enzymology, Research Centre for Natural Sciences). We verified the absence of Vps18 by WB and IC, and we selected two cell lines that were used for further investigations. After that, we established a Vps18 construct, which lacks the Rbsn binding site, and we investigated, which cellular processes has been affected. We examined the effect of the loss of Vps18 on autophagic markers, such as lipidation of microtubule-associated protein 1A/1B-light chain 3 (LC3) by WB. The conjugation of LC3 to the membrane component phosphatidylethanolamine (PE) occur during autophagy and it assigns autophagic structures such as phagophores, autophagosomes and autolysosomes. In the

case of failure in the completion of the degradation process -e.g.defect in autophagosome-lysosome fusion-, the level of the lipidated LC3 (LC3II) will increase. Both the lack of Vps18 (with the lack of HOPS complex) and chloroquine treatment (Mauthe et al.) cause defect in autophagosome-lysosome fusion, which elevates LC3II level. We found that the levels of lipidated form of LC3 (LC3 II) are elevated in the mutants, in the chloroquine-treated and in the starved HEK293 cells as well, compared to the control, non-starved HEK cells. To investigate the function of the Rbsn – Vps18 interaction, we generated a Vps18 variant, which lacks the Rbsn binding-site. We transfected Vps18 KO cells with full length (FL) or Rbsn-binding site mutant (ΔRbsnB) Vps18 constructs, and examined the level of lipidated LC3. FL Vps18 could rescue the elevated LC3II level to some extent, but the amount of the lipidated LC3 was still high. We observed similar elevated LC3II level in the control cells, transfected with empty vector, from which we concluded, that the transfection process itself elevates the level of lipidated LC3. To eliminate the effect of transfection on LC3II level, we generated Vps18KO cell lines that are stably transfected with FL or ΔRbsnB Vps18. We selected the transfected cells with hygromycin for two weeks, and then we examined the LC3II levels of the cell with WB. We found that both FL and ΔRbsnB Vps18 could decrease the level of the lipidated LC3II at the same extent.

Investigation of p62 and Lamp1 by immunocytochemistry, ultrastructural analysis

Next, we investigated the amount of another autophagic marker, p62 in our cell lines by IC analysis. p62 can ensure selectivity for the autophagic process, it has a LC3- and an ubiquitin-interacting domain as well, which provides selective binding of degradable material to the phagophore. We observed accumulation of p62 in Vps18 KO cells, which could be rescued with both FL and ΔRbsnB Vps18. We examined the level and distribution of the lysosome-specific Lamp1 protein, which showed elevated lysosome number in the Vps18 KO cells, due to the failure of proper lysosome maturation. FL Vps18 could reverse this process, while the ΔRbsnB Vps18 could not decrease the elevated Lamp1 level at the same degree as FL Vps18. We obtained the same results when we examined the ultra-structure of the cells by electron microscopy analysis. In the control HEK293 cells five multivesicular bodies (MB) and/or lysosomes per cell could be observed on the average, while it was 24 in the Vps18 KO cells. Eight MB and/or lysosomes per cells could be counted in the case of the FL Vps18 and 11 in cells transfected with ΔRbsnB Vps18.

Altogether, these results refer to the conclusion that the interaction between Vps18 and Rbsn is required for proper late endosome and lysosome maturation, while it is dispensable for autophagy.

In my application, I planned to use PLB-985, a myeloid leukemia cell line, which can be differentiated into neutrophil granulocyte or macrophage-like cells to establish Vps18 KO cell lines and investigate the effect of the loss of Vs18 on phagocytosis. However, only nucleofection seemed to be effective enough for further development of Vps18 KO cell lines; the commonly used liposome based transfection did not worked at all. Nucleofection requires specific instrument and transfection kit, which considered to be not cost-effective with low success-rate. Based on our results above, we decided to work further with HEK293 cells.

Actin network organization and integrin recycling of Vps18 KO HEK293 cells are altered

During the maintenance of the Vps18 mutant cell line we observed that the morphology of the cells are altered, they are round, and can not form the poligonic shape of the HEK293 cells. Based on earlier findings (Richardson et al.) Vps18 can interact with actin, which can affect cell motility and attachment. To reveal if Vps18 has an effect on actin organization, we examined the cytoskeleton with phalloidin staining. We observed that the actin network of Vps18 deficient cells are altered and they grow shorter projections than wild type cells. Vps18 KO cells stably expressing FL Vps18 showed normal morphology and projections, while ΔRbsnB Vps18 has significantly shorter projections compared to cells with FL Vps18.

Next we focused on the investigation of proteins required for cell adhesion. First, we examined the β1-integrin expression levels of the wild type and the Vps18 KO cells by western blot and IC. Based on the IC experiments there is no difference between the expression levels of β1-integrin and we could not obtain reasonable results with the same antibody in western blot. We wanted to investigate if the Vps18 KO cells could recycle the internalized integrin molecules back to the plasmamembrane properly, since it is an important part of the integrin turnover (Jonker et al.). Our data shows that in the absence of Vps18 cells can internalize integrins, but their integrin-recyling ability is altered: after 5 minutes significantly more β1-integrin positive dots could be observed in the Vps18 KO cells, compared to wild type. We investigated our stably transfected cell lines also, and we found that FL Vps18 could decrease the number of β1-integrin positive dots significantly, while the effect of ΔRbsnB Vps18 was not significant. Based on these we can conclude that the Vps18 – Rbsn interaction may plays role in the actin network organization and in the β1-integrin recycling.

Vps18 indirectly interacts with Vps45, possibly via Rbsn

According to earlier data from the literature (Nielsen et al.), Rbsn is a Vps45 binding protein. Vps45 is an SM protein that can bind SNARE proteins, which are required for proper fusion of endosomes. Since it seems that Vps45 and Rbsn plays role in β1-integrin recycling (Rahajeng et al.), we wanted to know, if Vps45 can be another interacting partner of Vps18 and Rbsn. We observed that both Vps18 and Rbsn could precipitate Vps45 efficiently in anti-FLAG coIP experiments. However, according to y2h analysis, Vps45 binds only Rbsn directly; the interaction with Vps18 is indirect.

To reveal, if Rbsn could bind Vps18 and Vps45 simultaneously, we narrowed further the Vps18 binding site of Rbsn. Earlier we mapped this site to the N-terminal part of the protein (aa 1-421) and we divided this N-terminal region further to three pieces. Amino acids 285-421 of Rbsn could bind Vps18, while the Vps45 binding site was mapped to aa 1-140 by y2h. This latter finding is in line with earlier data from literature (Rahajeng et al.), where aa 100-101 and aa 105-109 was proved to be the Vps45 binding site. Based on this information we destroyed this site with site directed mutagenesis and we investigated, if this mutant Rbsn form could still bind Vps18. We found that in spite of the destroyed Vps45 binding site, Rbsn still bind Vps18, hence the binding sites do not overlap. We concluded that Rbsn, Vps45 and Vps18 could form -at least transiently - a complex.

Summary

Taken together we identified new interactions among the tethering factors of the human endosomal system, namely interactions between Rbsn – Vps18 (HOPS/CORVET) and Vps45. Vps18 – Rbsn interaction is required for endocytic progression, while does not play role in autophagy. Additionally, it seems to have a function in actin network reorganization and integrin recycling -likely together with Vps45.

Conferences, personnel

In the 2019/2020 study year Ármin Sőth started his PhD under my supervision. During his MSc studies he took part in the XXXIV. Congress of Student Research Societies and he was third-placed in the Biology Sciences Section, Cellular Biology Division.

In the end of the second semester of 2019/2020 Molnár Márton III. BSc biology student defended his thesis under my supervision with excellent result. He continues his work in our lab and he presented our results in the Congress of Student Research Societies of ELTE University at 5th December, 2020. His work was suggested to be presented at the XXXV. Congress of Student Research Societies in 2021.

We presented our results in the "Hungarian Molecular Life Sciences" meeting in Eger between March 29-31, 2019. We intended to present our results in the 9th Proteasome and Autophagy Congress in Clermont-Ferrand, France, April 22-24, 2020 as well. However, due to the coronavirus pandemic, the conference was postponed, and after that it was cancelled.

Scientific publications

Beside the above mentioned scientific article (Simon-Vecsei et al.), one review article (I am co-first author) and three additional publication (I am co-author) was published during the period of the fellowship. Sum of the impact factors: 32,626.

Literature

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