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ApRab11, a cnidarian homologue of the recycling regulatory protein Rab11, is involved in the establishment and maintenance of the *Aiptasia–Symbiodinium* endosymbiosis ☆

Ming-Chyuan Chen [a], Ming-Cheng Hong [b], Yung-Sen Huang [c], Ming-Chin Liu [d]
Ying-Min Cheng [a], Lee-Shing Fang [d,*]

[a] *Department of Marine Biotechnology, National Kaohsiung Marine University, Kaohsiung 811, Taiwan, ROC*
[b] *Institute of Marine Biology, National Sun Yat-sen University, Kaohsiung 806, Taiwan, ROC*
[c] *Department of Life Science, National University of Kaohsiung, Kaohsiung 811, Taiwan, ROC*
[d] *Department of Planning and Research, National Museum of Marine Biology and Aquarium, Pingtung 944, Taiwan, ROC*

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Abstract

Endosymbiotic association of the *Symbiodinium* dinoflagellates (zooxanthellae) with their cnidarian host cells involves an alteration in the development of the alga-enclosing phagosomes. To uncover its molecular basis, we previously investigated and established that the intracellular persistence of the zooxanthella-containing phagosomes involves specific alga-mediated interference with the expression of ApRab5 and ApRab7, two key endocytic regulatory Rab proteins, which results in the selective retention of the former on and exclusion of the later from the organelles. Here we examined the role of ApRab11, a cnidarian homologue of the key endocytic recycling regulator, Rab11, in the *Aiptasia–Symbiodinium* endosymbiosis. ApRab11 protein shared 88% overall sequence identity with human Rab11A and contained all Rab-specific signature motifs. Co-localization and mutagenesis studies showed that EGFP-tagged ApRab11 was predominantly associated with recycling endosomes and functioned in the recycling of internalized transferrin. In phagocytosis of latex beads, ApRab11 was quickly recruited to and later gradually removed from the developing phagosomes. Significantly, although ApRab11 immunoreactivity was rapidly detected on the phagosomes containing either newly internalized, heat-killed zooxanthellae, or resident zooxanthellae briefly treated with the photosynthesis inhibitor DCMU, it was rarely observed in the majority of phagosomes containing either newly internalized live, or healthy resident, zooxanthellae. It was concluded that through active exclusion of ApRab11 from the phagosomes in which they reside, zooxanthellae interfere with the normal recycling process required for efficient phagosome maturation, and thereby, secure their intracellular persistence, and consequently their endosymbiotic relationship with their cnidarian hosts.

Keywords: Endosymbiosis; Endocytosis; Rab11; Phagocytosis; Zooxanthellae; Symbiotic sea anemone; ApRab11; Phagosome maturation; Intracellular survival; Endocytic recycling

Symbiotic dinoflagellates, commonly known as zooxanthellae, are a taxonomically diverse group of marine microalgae, predominantly classified under the genus *Symbiodinium*. They are well known for their ability to establish intracellular symbiosis (endosymbiosis) with numerous marine cnidarians such as jellyfish, sea anemones, and corals, and are directly responsible for the high primary productivity and biodiversity of tropical coral reefs worldwide [1–4]. In nature, zooxanthellae are seldom found free-living in the water column or sediments [1,5]. However, they are present in great numbers in the tissues of their cnidarian hosts amounting to several millions per square centimeter of host tissue area [1,6]. Considering the oligotrophic nature of a typical coral reef ecosystem, this drastically biased population distribution highlights

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* Corresponding author. Fax: +886 8 8825066.
E-mail address: lsfang@nmmba.gov.tw (L.-S. Fang).

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the central role of intracellular association of zooxanthellae with animal hosts in the acquisition of essential inorganic nutrients and subsequently in promoting the survival and propagation of their populations.

During endosymbiosis with their cnidarian hosts, zooxanthellae are exclusively situated in the digestive cells of the gastrodermal tissue layer, individually enclosed in a symbiosis-specific organelle, known as the symbiosome, where they grow and replicate [1,6,9]. These organelles are originating from phagocytic uptake of zooxanthellae by the host digestive cells [6–8], but unlike typical phagosomes containing food and other inanimate particles, they do not or seldom fuse with lysosomes [7]. This raises the question regarding the distinct properties of symbiosomes which separate them apart from regular phagosomes in terms of the fusogenicity with lysosomes. Early studies on this issue have mainly focused on two unique features of symbiosomes, namely the production and release of photosynthate, and the chemical composition of symbiosome membrane [7,10]. Although correlative evidence has been gathered to implicate their involvement in the inhibition of lysosomal fusion, the exact identities of the responsible molecules and the corresponding molecular mechanisms remain completely unknown.

More recently, it has been found that contrary to prior belief, newly formed phagosomes do not fuse readily with lysosomes [11,13]; instead, their competency to fuse with lysosomes is gradually acquired after a complex, sequential series of membrane fusion/fission events with the endocytic compartments [12–15]. Endocytosis is now known to constitute two overlapping but distinct pathways, one leading to the degradation of the internalized materials (the degradation pathway) in lysosomes, the other to the retrieval of membrane receptors back to plasma membrane [12]. Membrane fusion with early endosomes and then with late endosomes (the degradation arm of endocytosis) has been shown to allow maturing phagosomes to acquire new membrane and luminal molecules [12,13], while concurring membrane fission (the recycling arm of endocytosis) acts to remove surface proteins and membrane from phagosomes as they mature [16,17]. Considering the complexity and multi-step nature of phagosome maturation, a late event in the series like the phagosome–lysosome fusion would inevitably be prevented from happening when a defect in a prior step has occurred. Accordingly, the poor fusogenicity of symbiosomes with lysosomes could have been caused by a zooxanthellae-mediated interference in any of the earlier steps along the phagosome maturation pathway.

To investigate the above possibility, we have previously chosen to examine two small GTP-binding proteins of the Rab family, Rab5 and Rab7, due to their respective specific localization on the early and late endosomes, and their key roles in regulating vesicular transport leading to lysosome fusion [18–20]. We cloned their corresponding functional homologues, ApRab5 and ApRab7, in the symbiotic sea anemone *Aiptasia pulchella*, and demonstrated that although like their mammalian counterparts, sequentially and transiently associating with maturing phagosomes containing inert particles, ApRab5 is selectively retained in, while ApRab7 excluded from phagosomes containing newly acquired live zooxanthellae. Furthermore, we showed that symbiosomes (phagosomes containing resident zooxanthellae) displayed a strong affinity to associate with ApRab5 but not with ApRab7, which could be reversed when zooxanthellae were briefly treated with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) to damage their photosynthesis systems [21,22]. All these findings confirm our hypothesis that for the newly formed phagosomes and the symbiosomes, the normal pathway of phagosome maturation has been altered by their inhabiting zooxanthellae and therefore their further development to fuse with lysosomes is inhibited.

To further extend the analysis of zooxanthellae-mediated inhibition of phagosome maturation, here we investigated whether the poor lysosomal fusogenicity of phagosomes harboring zooxanthellae could be attributed to a recycling defect. To this end, we placed our attentions on a specific member of the Rab family, Rab11, which has been established as a key regulatory protein mediating endocytic recycling [23]. Through the molecular cloning and characterization of ApRab11, an *Aiptasia* homologue of Rab11, we found that ApRab11 functioned in the recycling of internalized transferrin, and it was rapidly recruited to the phagosomes containing either newly internalized inanimate particles or resident zooxanthellae briefly treated with a photosynthetic inhibitor, but not to those containing either newly acquired live zooxanthellae or untreated resident zooxanthellae. Based on these findings, we conclude that live and healthy zooxanthellae actively exclude ApRab11 from the phagosomes in which they reside, presumably to interfere with the normal recycling process required for efficient phagosome maturation, thereby contributing to the establishment and/or maintenance of their endosymbiotic relationship with their host animals.

Materials and methods

Cells and the animal. The experimental animal, *A. pulchella*, was maintained in laboratory aquaria on a 14/10-h light/dark cycle at irradiance levels of 80–100 photons m^{-2} s^{-1}. The animals were fed with newly hatched brine shrimp twice per week. Aquarium water temperature was maintained at around 26–28 °C using submerged heating device, and one-half of aquarium water was replaced with fresh filtered seawater (1-μm Millipore) one day after each feeding. Cultured mammalian cells (HeLa and COS7 cells) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin sulfate (100 μg/ml) at 37 °C, in a humid incubator with 5% CO_2.

Cloning of full-length ApRab11 cDNA and sequence analysis. The approach employed to clone the full-length cDNA for *A. pulchella* Rab11 protein was as described [21,22], except that specific nested PCR primers were designed based on the obtained Rab11 homologous *Aiptasia* sequences to PCR-amplify the missing 5′ and 3′ sequences. The nested primers used for 5′ RACE are 5′-2 (5′-CTCGGGAAGCG CGCCATTGTGTTGGT-3′), Rab11-r1 (5′-CTTTTGCCTCATCAGT TGG-3′), and Rab11-r2 (5′-TCAAGTGTTTAGCAATGTCGTAGA-3′).