# 行政院國家科學委員會專題研究計畫　期中進度報告

# 探討酵母菌小分子鳥糞嘌呤核甘三磷酸腺(ARLs)在囊膜傳遞的功能(2/3)

計畫類別：個別型計畫
計畫編號：NSC91-2320-B-002-071-
執行期間：91 年 08 月 01 日至 92 年 07 月 31 日
執行單位：國立臺灣大學醫學院分子醫學研究所

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報告類型：精簡報告

處理方式：本計畫可公開查詢

中　華　民　國 92 年 6 月 2 日

**Functional characterization of yeast small GTPases, ARF-like proteins (ARL), in vesicular trafficking**

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ADP-ribosylation factors (ARFs) were initially recognized and purified for their ability to stimulate the ADP-ribosyltransferase activity of the cholera toxin A subunit to Gsα. ARFs are members of the Ras superfamily of ~20Kda guanine nucleotide-binding proteins and have intrinsic GTPase activity. With numerous studying, ARFs are believed to participate in vesicle transport. As other members of the Ras superfamily, ARF with GDP bound is inactive and after the replacement of bound GDP with GTP produces active ARF-GTP, which can associate with membranes and recruit effectors. Both forms are important in vesicular transport, which requires that the ARF molecule cycle between active and inactive states. Under physiological conditions, release of GDP from ARF, the prerequisite for GTP binding, is very slow and is markedly accelerated by guanine nucleotide-exchange factors or GEFs, and the hydrolysis of bound GTP to yield ARF-GDP is similarly very slow in the absence of specific GTPase-activating proteins or GAPs.

ARF-like (ARL) proteins, although homologous to ARFs, can’t stimulate the ADP-ribosyltransferase activity. Seven human ARL and two yeast ARL genes have been identified. Although the results from yeast and mammalian ARL1 indicate that ARL1p has a role in Golgi structure and function and regulate specific membrane traffic, the function of most ARLs remains unclear. Not only function, but also the regulators of ARLs, GEFs and GAP, are also unknown.

Our research goal is to delineate the molecular mechanisms how yARL1 and yARL3 carry out their functions in intracellular vesicular transport.

The following studies are our goals in this proposal:

1. To characterize the structure and function of yARLs.
2. To identify and characterize molecules interacting directly with or functioning in conjunction with yARLs.
3. To study the molecular mechanisms of yARLs and their interacting molecules in regulating vesicular trafficking.

Progress:

At least five GAPs for ARF in yeast have been found, including Gcs1p, Glo3p, Sps18p, Age1p and Age2. Gcs1p was originally identified as a cold-sensitive mutant that failed to resume logarithmic growth from stationary phase. To date, Gcs1p is thought to be a GAP and have GAP activity to ARF1 and ARF2 proteins in vitro. Poon et al. suggest that Gcs1p and Age2p may mediate exocytic vesicular transport pathway from the TGN, and the retrograde Golgi–to-ER pathway is mediated by the Gcs1p and Glo3p pair. In addition to GAP activity, Gcs1p is also reported to be required for normal actin cytoskeletal organization and for actin polymerization in vitro. Although only ARF1p and ARF2p have been mentioned to be substrates of Gcs1p, the multiple functions of Gcs1p imply that it must have multiple roles or function at multiple places.

From the studying of yeast ARF1, Eugster et al. reported that only the truncated active ARF1p can interact with GAPs, Gcs1p and Glo3p, we designed the N-terminal truncated active yeast ARL1p (ARL1　17NQ72L) to fish out its GAP. With yeast two hybrid experiment, we find Gcs1p can also interact with ARL1　17NQ72L. Their

direct-interaction was further proved by *in vitro* binding assay, and in *vivo* pull down analysis.

Although from these binding assay, ARF1p show similar binding affinity to Gcs1p than ARL1p does, the GAP activity assay show that Gcs1p has stronger enzymatic activity to ARL1p than ARF1p. Recombinant Gcs1p has stronger GAP activity to rARL1p than to rARF1p. In contrast, rGlo3p has much higher GAP activity to rARF1p than rARL1p.

The interaction and GAP activity of Gcs1p to ARL1p indicate that Gcs1p may be GAP of ARL1p. If this is true, the overexpression or deletion of Gcs1p should affect the guanine nucleotide-binding status of ARL1 in vivo. Since ARFs are thought to associate with membrane when it is GTP bound form, indirect immonofluorescence staining was used to detect endogenous ARL1p localization. The punctate signals of ARL1p suggested that ARL1p was localized to Golgi, however the signal did not co-localize with some Golgi localizing proteins, such as: the medial- or late Golgi protein Emp47p and the late Golgi protein Sec7p. Interestingly, ARL1p signals were partially co-localized with late Golgi protein Sft2p. These results indicated ARL1p was localized at a sub-region of late Golgi, which is distinct from Sec7p and Emp47p.

Next, we take advantage of the late Golgi localization of ARL1p to determine the effect of Gcs1p on ARL1p. We find that the overexpression of Gcs1p cause the punctate distribution of ARL1p to become diffuse, suggesting that active GTP form of Arl1p was catalyzed to inactive GDP form. The Golgi localization signal of Arl1p is enhanced and cytoplasmic signal is decreased in *gcs1* knockout yeast, indicating that Moreover, the effect of Gcs1p overexpression on ARL1p localization is dependent on its GAP activity, since overexpression of the zinc-finger disrupted Gcs1p doesn't change ARL1p localization. These data indicate that Gcs1p may be GAP of ARL1p in vivo.

We have found that gcs1 deletion would cause mild delay of ALP (Alkaline phosphatase) transport from Golgi to vacuole. Similar to gcs1 deletion yeast, arl1 deletion yeast also show mild delay of ALP maturation and the gcs1arl1 double mutant yeast have no synergic effect on ALP maturation indicate that these two proteins may participate in the same pathway. However, the mild effects imply this pathway is not the major function of ARL1p and Gcs1p, this phenotype may come from side effect.

Although we still have not found the actual traffic pathway that ARL1p and Gcs1p regulated, our preliminary data indicate ARL1p may cross talk with Ypt6p retrograde pathway since gcs1arl1 double mutant yeast show Snc1p recycling defect. Besides, The ARL1p-interacting protein Imh1p is a multi-copy suppressor of ypt6 and arl1ypt6 or imh1ypt6 double mutants are synthetic lethal.

Since membrane traffic is so complex and dynamic, we should not make conclusion too early. The Snc1p recycling defect might come from defect of anterograde or others. Although Wang et al. reported Gcs1p might participate in endocytosis, we couldn't observe defect of FM4-64 endocytosis in *gcs1, arl1*, or *gcs1/arl1* double mutant at 16°C or 30°C. However, vacuole fragmentation is observed in *arl1* single mutant but not *arl1gcs1* double mutant. The fragmented vacuole in *arl1* mutant yeast was also observed by Bonangelion et al. This phenotype may imply that ARL1p is required for membrane traffic required for vacuole morphogenesis and Gcs1p has functional relationship with ARL1p.

We observed that ARL1p did not localize to Golgi complex in *arl3* mutant cell. It will be interesting to examine whether ARL3p can affect ARL1p through recruitment of GEF or other proteins or indirectly through change the membrane dynamic of Golgi. Thus, the relationship between ARL1p and ARL3p still need to be further

investigated. Do ARLs form a regulatory cascade like mammalian Rab proteins? Does ARL3p regulate ARL1 by recruiting its GEF? What's the functional relationship between ARL1p and Ypt6p? We'll try to answer these questions to uncover the functional roles of ARL1p and ARL3p in vesicular trafficking.