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Functional characterization of human ARL1 involved in intracellular trafficking: implication in lysosomal disorder

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As sequencing of the human genome nears completion, the genes that cause many human diseases are being identified and functionally described. This has revealed that many human diseases are due to defects of intracellular trafficking. There are several diseases that apparently affect the transport machinery for lysosomes. The broad, long-term objective of this proposal is to understand how proteins regulate vesicular transport. We would like to demonstrate that hARL1 and its effectors play an important role in intracellular vesicular transport from trans-Golgi to lysosome, and thereby affect cellular function. We will employ molecular genetics, biochemistry, and cell biology to test this hypothesis.

The following studies are our goals in this proposal:

1. To identify and characterize proteins interacting directly with or functioning in conjunction with ARL1 in lysosome trafficking.
2. To characterize structure and function of ARL1 protein involved in lysosome trafficking.
3. To study the molecular mechanisms of ARL1 interacted with its effectors.

Abstract

To identify molecules that might act as down-stream effectors of hARL1, we used hARL1(Q/L) as bait in a yeast two-hybrid screen of human fetal liver cDNA library. 43 positives were obtained and several distinct genes were chosen for further analysis. Among them, golgin-245 (9 clones), ApoC2 (8 clones), Arfaptin 2 / POR1 (4 clones), ApoB (2 clones), and Sec10 (one clone) will be further characterized to support the their functional interactions. We also constructed wild type hARL1 and hARL1-T/N to test whether interaction of hARL1 and their interacting proteins is nucleotide-dependent. We also found that hARL1 and golgin-245 bound to Golgi membranes, consistent with a function in vesicular trafficking. Expression of putatively constitutively active mutant of hARL1, ARL1(Q71L), in cells led to down-regulate golgin-245 bound to Golgi membranes. *In vitro* protein-interaction assays showed that hARL1(Q71L) interacted with C-terminal of golgin-245. How is sorting signal recognition regulated so that interaction with each

vesicle-receptor binding protein occurs only at the appropriate organelle? Cooperative association of cargo, vesicle binding proteins, and additional factors into mutimeric, trans-Golgi and/or lysosome-specific complexes will be investigated in this project.

Introduction

ADP-ribosylation factors (ARFs) are a family of highly conserved, ~20-kDa guanine nucleotide-binding proteins which were originally identified and purified by their ability to enhance the ADP-ribosyltransferase activity of cholera toxin, comprise a distinct subfamily of Ras-related small GTP-binding proteins and have been found in all eukaryotic cells from yeast to human (reviewed by Boman and Kahn, 1995; Moss and Vaughan, 1998). ARF-like proteins (ARLs) are structurally divergent, with sequences 40-60% identical to any ARF or to each other. ARLs, unlike ARFs, usually do not activate cholera toxin ADP-ribosyltransferase, although hARL1 had relatively low activity (Hong et al., 1998). ARLs differ also from ARFs in having demonstrable GTPase activity and different conditions that favor guanine nucleotide binding (Moss and Vaughan, 1998). To date, the ARF family comprises at least six ARFs and six (ARL) proteins. They can be divided into three classes based on deduced amino acid sequence, protein size, phylogenetic analysis, and gene structure (Boman and Kahn, 1995; Moss and Vaughan, 1998). ARF famiy protein can cycle between active GTP-bound and inactive GDP-bound forms that bind selectively to effectors, guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs). ARFs have recently been recognized as regulators of intracellular vesicular transport. ARF1 belong to Class Ⅰ ARFs the have been most extensively studied and shown to regulate the assembly of coat protein complexes onto vesicle budding sites including the COPI complex on the Golgi complex, the AP-1 clathrin adaptor complex on the *trans*-Golgi network(TGN), and the AP-3 complex on endosome (Hirst and Robinson, 1998; Lippincott-Schwartz et al., 1998).

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The biological functions of ARLs are still unclear. The first member (ARL1) was originally cloned from *Drosophila* (Tamkun et al., 1991) and is essential for survival. ARLl was localized in the Golgi complex of normal rat kidney cells (Lowe et al., 1996) and *S. cerevisiae* (Lee et al., 1997), consistent with a function in vesicular trafficking. Unlike *Drosophila* ARL1, knock-out of the yeast ARL1 gene was not lethal (Lee et al., 1997). ARL2 regulates the interaction of tubulin-folding cofactor D with native tubulin (Bhamidipati *et al.*, 2000) and ARL4 and ARL5 may function in the nucleus (Lin *et al.*, 2000; Lin et al., 2002). To obtain additional clues to physiological role(s) of ARL1, we investigated the expression, subcellular

localization, and possible downstream effectors of ARL1.

Methods and materials

Expression plasmid

The open reading frame of human ARL1 was obtained by polymerase chain reaction using primers that incorporated NdeI or EcoRI site at the initiating methionine and XbaI sites at the 6 base pairs downstream the stop codon, respectively. Full-length wild type hARL1 cDNA was generated using 5' (sense) primer ARL1A or ARL1B, and 3' (antisense) primer ARL1C (Tabel 1). Replacement of Thr31 with Asn (T31N) and Gln71 with Leu (Q71L) were accomplished using a two-step PCR technique as described. The 5' (sense) mutagenic primer ARL1D and the antisense mutagenic primer ARL1E were used to generate hARL1T31N. The 5' (sense) mutagenic primer ARL1F and the antisense mutagenic primer ARL1G were used to generate hARL1Q71L. Using 5' (sense) mutagenic primer ARL1H to replace of Gly2 with Ala to generate ARL1G2A. The point mutation is underlined in oligonucleotide sequences (Tabel 1). To generate ARL1d17NQ71L with deletion of 17 amino acids at N terminus, the 5' (sense) primer ARL1I or ARL1J was used. All ARL1 PCR fragments were ligate to the pSTBlue-1 vector (Novagen) first and then they were cloned into the mammalian expression vector pcDNA3.1A between the EcoRI and XbaI sites. For expression ARL1 in E. coli, all fragments were cloned into pET15b vector (Novagen) between NdeI and BamHI sites. To make LexA BD-ARL1 express in yeast system, ARL1 fragment were cloned into pBTM116 vector at EcoRI site.

Golgin-245C (residues 1846-2229), Golgin-245CÄC (residues 1846-2032) and Golgin-245CÄN (residues 2033-2229) were generate by PCR from cDNA clone in pACT2 using the primers Golgin4.1, Gogin4.2, Golgin4.3 and Golgin4.4. VP16 fusion Golgin-245 fragments were generate by inserting into pVP16 at the NotI site. All PCR products were purified, subcloned, and sequenced by the dideoxy chain termination method.

Antibodies

Peptide corresponding to the residues 130-145 of hARL1 (MEQAMTSSEMANSLGL) were synthesized with a Cys residue attached to the C terminus and conjugated to keyhole limpet hemocyanin (KLH) carrier protein. Recombinant GST-Golgin-245 C (residues 1846-2229) and GST-arfaptin 2 was purified as described above. Rabbit were immunized with KLH-conjugated peptide and purified Golgin-245C and arfaptin 2 protein to get antisera. Other primary antibodies used were monoclonal mouse anti-myc (9E10)(Berkeley antibody company), monoclonal mouse anti-Golgi 58K protein (Sigma) and monoclonal mouse anti-βCOP (Sigma). Immunoblotting bands patterns was detected with HRP-conjugated goat anti-rabbit or goat anti-mouse

IgG antibody (Amersham Pharmacia Biotech), followed by ECL reaction (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The immunofluorescence staining pattern were detected with Alexa 594-conjugated or Alexa 488-conjugated secondary antibodies (Molecular Probes Inc.).

Yeast Two-hybrid Screen and Assay

Yeast reporter strain L40 (MATa HIS3 200 trp1-901 leu2-3, 112ade2 LYS2::(lexAop)4-HIS3 URA3::(lexAop)8-lacZ GAL4), plasmids (pBTM116 and pVP16), and human fetal liver cDNA library in pACT2 (Clontech) for the yeast two-hybrid screen were the generous gift from Dr. H. Shih(National Health Research Instiutes, R.O.C.,Taiwan). Yeast strain AMR70 (MATáHIS3 lys2 trp1 leu2 URA3::(lexAop)8-lacZ GAL4) , which is used for testing specificity of interacting by mating was obtained from Dr. Yen, J.J.Y. (Academia sinica, R.O.C., Taiwan). The yeast strains are constructed with two readouts for an interaction of histidine auxotrophy and â-galactosidase expression with the use of the LexA DNA binding domain and GAL4 activation domain system. Yeast strains were grown at 30°C in YPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) or in synthetic minimal medium with appropriate supplements. For express LexA fusion protein of hARL1, hARL1Q71L, hARL1T31N, hARL1WTd17N and hARL1d17NQ71L in yeast, we constructed these clones, respectively, by inserting PCR-generated fragments into the EcoRI site of the pBTM116 plasmid. Control plasmids for express LexA-ARL2, LexA-ARL3, LexA-ARL4 and LexA-ARL5 have been described previously. The expression of each LexA fusion protein was confirmed by immunoblotting.

For two-hybrid screening, the yeast reporter strain L40, was transformed with a plasmid encoding the activated ARL1 mutant construct ARL1Q71L and a human fetal liver cDNA library in pACT2 (1.2×10^6 transformants) by the lithium acetate method and double transformants were selected for histidine auxotrophy on minimal medium lacking histidine. Colonies of histidine auxotrophy were patched on selective plates and assayed for â-galactosidase activity by a filter assay. Colonies of yeast transformants were transferred to nitrocellulose membrane and permeabilized in liquid nitrogen. Each membrane was placed on a Whatman No.1 filter paper that had been presoaked in Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCI, 1 mM $MgCl_2$) containing 0.33 mg/ml 5-bromo-4-chloro-3-indolyl-â-D-galactoside. Filters were incubated at 30 ℃ for 30 min to 6 h, with color scoring at regular intervals. Obvious blue color development within 6 h was considered positive. The GAL4-AD-cDNA hybrid in pACT2 vector were isolate from colonies displaying a HIS+/lacZ+ phenotype by phenol extraction and introduced into HB101-compentent

cell by electro-transformation. These were then tested in two-hybrid assay in L40 strains carrying lamin as partner to confirm the specificity of the interaction with ARL1Q71L. To enrich for potential ARL1 effectors or GTPase-activating proteins (GAPs), each library plasmid was also transformed into yeast AMR70 and then mating with the yeast L40 strain containing the appropriate pLexA-lamin, pLexA-ARL1, pLexA-ARL1Q71L, pLexA-ARL1WTd17N, pLexA-ARL1Q71Ld17N, pLexA-ARL1T31N, pLexA-ARL2, pLexA-ARL3, pLexA-ARL4, pLexA-ARL4Q79L, pLexA-ARL5, and pLexA-ARL5(Q80L), to test for specificity and then sequence.

Expression and Purification of Recombinant Proteins

The entire open reading frame of human ARL1 was obtained by PCR, using primers that incorporated unique NdeI sites at the initiating methionine. For preparation of the His-tagged fusion protein, the PCR product was cloned into the expression vector pET15b (Novagen) at the NdeI and BamHI site. The entire sequence of the ARL1 constructs was confirmed by automated sequencing. For production His-hARL1 protein, the pET15b-ARL1 constructs were transformed to BL21 (DE3). Bacterial pellets were harvested and then incubate with lysozyme for 30 min at 4 °C. Subsequently cells were disrupted by sonication and centrifuged (10,000 xg at 4 °C for 30 min) to separate soluble fraction (supernatant) and insoluble fraction (pellet). His-tagged fusion protein was isolated on Ni2+-NTA resin (Qiagen, Chatsworth, CA) by manufacturer's instructions. The purity of the His-tagged hARL1 protein was assessed by SDS-PAGE.

Glutathione-S-transferase (GST) fusion proteins, GST-Golgin-245C, GST-Golgin-245CÄC and GST-Golgin-245CÄN, were synthesized in mid-log phase E. coli BL21(DE3) by induction with 0.5 mM isopropyl-D-thiogalactopyranoside (IPTG) for 3 hours at 37 °C. Bacterial pellets were dispersed in cold PBS (pH7.4, containing 2.5 μ g/ml pepstatin A, 2.5 μ g/ml aprotinin, 2.5 μ g/ml antipain, 5 μ g/ml Nα - ρ -tosyl-L-lysine chlorometyl ketone, 2.5 μ g/ml leupeptin, and 5 μ g/ml benzamidine). The soluble form of E. coli expressing GST fusion protein were incubated with glutathionine-Sepharose 4B beads (Amersham Pharmacia biotech) at 4 °C for overnight. Then the beads were washed five times with cold PBS (pH 7.4), and resusupend in cold PBS finally. GST and GST-Golgin-245C, immobilized on glutathionine-Sepharose 4B beads were quantified by SDS-PAGE with Coomassie Blue staining.

In vitro GST pull down assay

Each pET15b-hARL1 constructs were express in E. coli, and each soluble fraction

were incubate with 10 ì g GST or GST-Golgin-245 C (residues 1846-2229) immunized on glutathionine-Sepharose 4B beads in PBS for 1 hour at room temperature. After washing five times with ice-cold washing buffer (25 mM Tris-HCl, pH8.0, 100 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol), add 20 ì l SDS-sample buffer to the washed beads, boiled for 10 minutes at 95 °C. Protein binding result were analysed by SDS-PAGE and immunobloting.

Cell Culture and Transient Transfection

COS7 cells (ATCC: CRL-1651) were grown in Dulbecco's modified Eagle's medium (DMEM, GIBCO), supplemented with 10 % fetal bovine serum and 100 units/ml each of penicillin and streptomycin at 37 °C. The cDNA fragments of hARL1 or its mutants were fused in-frame to the N-terminus of myc by subcloning into pcDNA3.1/Myc-His A (Invitrogen) at the EcoRI and Xba I sites. Cells were seeded on culture dish 16 h before transfection with the aid of Lipofectamine (Invitrogen) according to manufacturer's procedures. Medium was replaced with fresh growth medium the day after transfection, and cells were harvested 30 to 36 h later for analysis.

Indirect Immunofluorescence Staining

5 x 104 COS-7 cells were cultured on glass coverslips in 12-well plate and transfect with 1ì g plasmid and 2.8ì l Lipofectamine. 48 h after transfection, cell were fixed with 4% formaldehyde in PBS for 15 minutes, incubated with 0.1% Triton X-100 and 0.05% SDS in PBS for 5 mins to permeabilize cells, and then block cells in blocking solution (0.1 % saponin, 0.2 % BSA in PBS) for an additional 30 minutes. Cells were incubated with primary antibodies in blocking solution for 90 minutes. After extensive washing with PBS, cells were incubated with the Alexa 594-conjugated or Alexa 488-conjugated secondary antibodies (Molecular Probes) in blocking solution for 1 hour, washed three times with PBS, mounted in 90 % glycerol in PBS containing 1 mg/ml of ñ-phenylenediamine (supplemented with Hoescht 33258), and then observed with the Nikon Microphot SA microscope and photographed with Kodak Tri-X 400 film.

LysoTracker colocalization studies

Coverslips with transiently transfected human ARL1T31N COS cells were incubated with the acidotropic dye LysoTracker Red DND-99 (Molecular Probes Inc.) 50 nM diluted in DMEM containing 10 % FCS for 40-60 mins at 37 ℃ to label lysosome, and then fixed with 4 % formaldehyde in PBS. Cells were permeabilized with ice-cold 100% acetone for 8 min, washed with PBS, and blocked with blocking solution (0.1

% saponin, 0.2 % BSA in PBS). Cells were incubated with monoclonal mouse anti-myc (9E10)(Berkeley antibody company) for 1 h to visualize the expressed ARL1T31N-myc (left panel), and processed for immunofluorescence microscopy.

Immunoprecipitation

For confirm hARL1 can interact with endogenous Golgin-245, cells transfected with hARL1 were washed with PBS and lysed in lysis buffer (50 mM Tris-HCl(pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxy cholate, 2.5 μg/ml pepstatin A, 2.5 μg/ml aprotinin, 2.5 μg/ml antipain, 5 μg/ml Nα-ρ-tosyl-L-lysine chloromethyl ketone, 2.5 μg/ml leupeptin, and 5 μg/ml benzamidine) for 30 minutes at 4°C. Cell lysates were centrifuged (15 minutes, 15,000 xg) at room temperature in a microcentrifuge, to remove nuclei and supernatants were saved for immunoprecipitation. Pre-clear the lysate with protein A-Sepharose by gently shaking 1 hr at 4 ℃. And then 10 μl of anti-myc monoclonal antibody (9E10) was added to the pre-cleared lysate. After rocking at 4°C for 6 hours, add 30 μl of protein A-Sepharose, and the samples were tumbled overnight at 4°C. Beads were pelleted by centrifugation (30 seconds, 600 xg) and washed five times with lysis buffer. The immunoprecipitates were then boiled at 95 ℃ for 10 min, chilled on ice and subjected to SDS-PAGE.

Table I Oligonucleotides used in the analysis of hARL1

Primer Name	Sequence (5'→ 3')
ARL1A	CAT ATG GGT GGC TTT TTC TCA AGT ATA TTT
ARL1B	GAA TTC GGT GGC TTT TTC TCA AGT ATA
ARL1C	TCT AGA CTG TCT GCT TTT TAA TGT TTC
ARL1D	TTA GAT GGA GCA GGA AAA AAC ACA ATT TTG TAC AGA TTA
ARL1E	TAA TCT GTA CAA AAT TGT GGT TTT TCC TGC TCC ATC TAA
ARL1F	GTC TGG GAT TTA GGA GGA CTG ACA AGT ATC AGG CCA TAC
ARL1G	GTA TGG CCT GAT ACT TGT CAG TCC TCC TAA ATC CCA GAC
ARL1H	GAA TTC GCC ATG GCT GGC TTT TTC TCA AGT ATA TTT TCC
ARL1I	GAA TTC GCC ATG AGA ATT TTA ATT TTG GGA TTA G
ARL1J	CAT ATG GCC ATG AGA ATT TTA ATT TTG GGA TTA G
Golgin4.1	GGA TCC TGG AAC TAA CCT GTC AGA TTT TG
Golgin4.2	GAA TTC TCA GAA GAT ACC ACT GCG AGG
Golgin4.3	GGA TTC TGG AAA GCC ATC AAG AAG AGA CA
Golgin4.4	GAA TTC TCA TAA AAG TCC AGC CTC CAC CT

Result

Identification of molecules that might act as down-stream effectors of hARL1

For further understanding the possible function of hARL1, we used the yeast two-hybrid screen to identify molecules that might act as binding partners of hARL1. The putatively constitutively active mutant of human ARL (hARL1Q71L) fused at their amino-terminus with the DNA binding domain of LexA as bait to screen human fetal liver cDNA library (Clontech). The bait caused no intrinsic transcriptional activation of the reporters. We get about 43 positive clones from 1.2 x 10^6 transformants. The DNA sequence of each library insert was determined and summary to nine proteins. They include 9 clones of Golgin-245(accession number U31906), 9 clones of Apolipoprotein C-Ⅱ(ApoCⅡ)(accession number NM_000483), 4 clones of Arfaptin 2 (Partner of RAC1) (POR1 protein) (accession number P53365), 2 clones of Apolipoprotein B(ApoB)(accession number AH003569), one clone of brain secretory protein hSec10p (HSEC10) (accession number U85946), and one clone of calcium and integrin binding 1 (calmyrin) (CIB1)(accession number NM_006384). We will be further characterized their interactions if having functional significant.

hARL1 specifically interacts with Golgin 245 at its C-terminal GRIP domain

Golgin-245 (also called p230 or Golga4) was found from a Sjogren's syndrome patient serum. The serum immunoprecipitated a 230-kD protein that was specifically localized to the trans-face of the Golgi stack and sensitive to brefeldin A. Golgin-245 can be detected in several cell types and species (Erlich et al., 1996; Fritzler et al., 1995). Current research (Cowan et al., 2002) found the message was highly upregulated in oligodendrocyte precursors at a stage just prior to myelination. Its amino acid sequence revealed the presence of multiple coiled-coil motifs, a sequence signature characteristic of the granins family of acidic proteins present in secretory granules of neuroendocrine cells, and sequence similarity to the heavy chain of myosin (Kooy et al., 1992). Further the amino acid sequence analysis (Munro and Nichols, 1999; Brown et al., 2001; McConville et al., 2002) found a novel Golgi-targeting domain (referred to as GRIP), of about 42 amino acids located at its C-terminus. The localization mediated by this domain is regulated by GTP γ S and brefeldin A(Kjer-Nielsen L, et al, 1999; Brown et al., 2001).

The clones of Golgin-245 which we get from library screen were encoded amino acids range from 1846 to 2229. The longest fragment of Golgin-245 clones (amino acid 1846-2229) was named Golgin-245C. Thus, the interaction of Golgin-245 and

ARL1 appeared to involve its C-terminus. We also used yeast two-hybrid assay to identify whether the interaction between Golgin-245 and hARL1 is nucleotide-dependent and specificity. Yeast transformants containing interacting proteins that transactivate two reporter genes, HIS3 and LacZ, exhibit â-galactosidase activity and can grow on minimal medium lacking histidine. As illustrated in Table 2 and Figure 1, LexA-hARL1Q71L, but not LexA-hARL1T31N, LexA-hARL1, LexA-hARL2, LexA-hARL3, LexA-hARL4, LexA-hARL4Q79L, LexA-hARL5, or LexA-hARL5Q80L, can interacted with the Gal4AD-Golgin-245C fusion protein and activated the reporter genes. So Golgin-245 does not interact with class Ⅱ of ARL (ARL2 and ARL3) and class Ⅲ of ARL (ARL4 and ARL5). The interaction is specific to ARL1 and nucleotide-dependent.

Interestingly, when we delete the N-terminal 17 amino acid of hARL1, either hARL1 Q71Ld17N or hARL1WTd17N can interact with Golgin-245C. It should be noted that positively charged residues in the N terminus have been postulated to be important for membrane binding by participating in electrostatic interactions with the lipid bilayer (Amor et al., 1994; Antonny et al., 1997). We suggest hARL1WTd17N can interact with Golgin-245C may be the result of conformation effect of the deleted N-terminal residues 1-17 of hARL1.
The GRIP domain of Golgin-245 is included in amino acid 1846-2229. To map the interaction region for hARL1 in Golgin-245 if involve the GRIP domain, we construct the deletion mutants of Golgin-245C. They are Golgin-245C△C (amino acid 1846-2032) and Golgin-245C△N (amino acid 2033-2229) (Fig.1A). The results from our yeast two-hybrid assay showed that the GRIP domain in Golgin-245 is essential for hARL1 association (Fig.1B).

We also performed GST pull-down experiments to confirm the results with the yeast two-hybrid assay. Recombinant His-hARL1 and its His fusion mutants were incubated with GST-Golgin-245C, GST-Golgin-245C△C or GST-Golgin-245C△N immobilized on glutathione Sepharose. As shown in Figure 2, only fragment of the recombinant GST-Golgin-245 containing GRIP domain, but not GST, associate with the hARL1 and hARL1Q71L. His-ARL1T31N and His-ARL4 don’t interact with GST-Golgin-245. Base on the *in vitro* pull down assay hARL1WT can direct interact with Golgin-245. It was different from the result by yeast two-hybrid assay. We assume that it maybe lack post-translation modification of ARL1 in bacteria result in ARL1 prefer GTP-bound form in bacteria. Meanwhile, we observe that one smaller protein fragment exist in our reaction solution with stronger interaction to Golgin-245. This protein fragment could be detected by our ARL1 antibody, but not by His

antibody, suggest it may be N-terminal degraded form of hARL1. Extract the protein fragment from acrylamide gel and sequence by Edman degradation show it was the deleted N –terminal residues 1-17 of ARL1.

Cross-reaction between human ARL1 and yeast ARL1

The homologue of Human ARL1 in yeast *S. cerevisiae* is yARL1. The genome of the yeast *S. cerevisiae* encodes only one protein with a GRIP domain, namely the coiled-coil protein Imh1p. The IMH1 gene was originally isolated as a suppressor of the temperature-sensitive lethality of a mutation in the small GTP-binding protein Ypt6p, a close relative of the mammalian protein Rab6 (Li et al., 1996). Using yARL1QL as bait to screen yeast cDNA library we obtained Imh1p (Liou et al., unpublish data). Because Golgin-245 and Imh1p share several characteristics, such as GRIP domain, we want to known whether Golgin-245 interact to yARL1 and vice versa. Identified by yeast two-hybrid assay, only GTP bound form of ARL1, either human or yeast, can interact with GRIP domain-containing Golgin-245 or Imh1p (Fig.). So we suggest that we can use yARL1 as a model in yeast system to further investigate the physiological function of ARL1.

ARF effector arfaptin-2/POR1 can interact with ARL1

Using a yeast two-hybrid screen of an HL-60 cDNA library with an ARF3Q71L mutant as bait, Kanoh et al.(1996) obtained cDNAs encoding arfaptin 1 and arfaptin 2. Arfaptin 1 localize in Golgi complex when overexpress in COS cells (Kanoh et al., 1996). The deduced amino acid sequence of arfaptin 2 was 60% identical and 81% homologous to that of arfaptin 1 when consensus substitutions were included. Arfaptin 2/POR1 has been shown to interact with the activated forms of ARF1, ARF3, and ARF6 (Kanoh et al., 1997; Van Aelst et al., 1996; D'Souza-Schorey et al., 1997). It proposed that arfaptin 2/POR1 is a binding protein for GTP-ARF acting in Golgi complex. Northern blot analysis revealed ubiquitous expression of a 2.1-kb transcript that was relatively higher in liver, pancreas, and placenta. Arfaptin 2/POR1 was cloned independently by the other research groups as a binding protein for GTP-Rac1 (POR1) (Van Aelst et al., 1996). It was proposed that arfaptin 2/POR1 is involved in membrane ruffling through cytoskeletal rearrangements at the plasma membrane. Arfaptin 2/POR1 was a common downstream effector for both ARF1 and Rac1. It suggest that arfaptin 2/POR1 may be a common downstream effector for both ARF1 and Rac1 and involved in interactions between the ARF and Rho signaling pathways (Tarricone et al., 2001). Recently, arfaptin 2/POR1 was also found to be a regulator of Huntingtin protein aggregation possibly by imparing proteasome function (Peters et al., 2002).

Using hARL1Q71L as bait to screen library, one of the interacting protein we obtain is arfaptin 2/POR1, as previous finding (Hillary et al., 1999; Lu et al., 2002). We use yeast two-hybrid assay to further confirm the interaction of ARL1 and arfaptin 2/POR1 if specificity and dependent on nucleotide binding. Like Golgin-245, the interaction of arfaptin 2/POR1 and ARL1 is specificity and depend on nucleotide. VP16-Arfaptin 2/POR1 interact with LexA-hARL1WTd17N, LexA-hARL1Q71L and LexA-hARL1Q71Ld17N, but not LexA-hARL1T31N, LexA-hARL1, LexA-hARL2, LexA-hARL3, LexA-hARL4, LexA-hARL4Q79L, LexA-hARL5, or LexA-hARL5Q80L. Arfaptin 2/POR1 also interacts to ARF1Q71L as previous finding (Kanoh et al., 1997). We also test the interaction of arfaptin 1 and ARL1. Surprisingly, Arfaptin 1 can interact with hARL1Q71L weakly, but not interact with hARL1Q71Ld17N. Meanwhile, arfaptin 1 desn't interact to ARF1Q71L different from other group (Kanoh et al., 1997) in our yeast two-hybrid system (Fig.).

Other possible binding partner of hARL1

Two of hARL1 possible interacting partner are ApoC2 and ApoB by our yeast two-hybrid library screen.. Apolipoproteins and their receptors are the main controllers of lipid metabolism. Human ApoCs are protein constituents of chylomicrons, VLDL, and HDL. Apolipoprotein C-II (ApoC2) is a necessary cofactor for the activation of lipoprotein lipase, the enzyme that hydrolyzes triglycerides in plasma and transfers the fatty acids to tissues. The importance of ApoC2 has unequivocally been demonstrated in patients with genetic defects in the structure or production of apoC2, all of whom display high circulating levels of triglycerides (TGs) and are phenotypically indistinguishable from patients with LPL deficiency (John et al., 1999). ApoC2 is synthesized with a 22-residue signal peptide that is cleaved cotranslationally in the rough endoplasmic reticulum (Sharpe et al., 1984). The remaining single polypeptide chain of 79 amino acid residues has a calculated molecular mass of 8.8 kDa (Fojo et al., 1984). The structure of ApoC2 is predicted to contain 3 helical regions which are thought to be involved in phospholipid binding (Captano et al., 1979; Kinnunen et al., 1977). To test whether ApoC2 specifically interact with hARL1, not to other ARF and ARL, we performed yeast two-hybrid assay. Unlike to Golgin-245 and arfaptin 2/POR1, Gal4 AD-ApoC2 only interacts to full length LexA-hARL1Q71L, not to LexA-hARL1, LexA-hARL1d17N, LexA-ARL1Q71Ld17N, LexA-hARL1T31N, LexA-hARL2, LexA-hARL3, LexA-hARL4, LexA-hARL4Q79L, LexA-hARL5, LexA-hARL5Q80L, LexA-hARF1 or LexA-hARF6. It suggests that the residues 1-17 of ARL1 may be require for hARL1 and ApoC2 association and their interaction is

nucleotide-dependent. We also assume that the N terminal region in hARL1 isn't essential for Golgin-245 and arfaptin 2/POR1. When deleted N terminalresidues 1- 17 of ARL1, either hARL1WTd17N or hARL1Q71Ld17N have stronger interaction than full-length hARL1WT and hARL1Q80L. We will further investigate the specificity and significance of ARL1 interaction with other proteins.

Characterization of antibodies recognizing golgin245 or arfaptin2

In order to examine whether golgin245 and arfaptin2 are functionally related to Arl1, we used recombinant as antigens to immunize rabbits. Anti-golgin245 and anti-arfaptin2 antibodies could recognize recombinant GST-golgin245C' and GST-arfaptin2, respectively. Perinuclear Golgi-like staining could be detected in COS-b cells processed to indirect immunostaining probed with anti-golgin245 or anti-arfaptin2. Antiserums were depleted by GST-golgin245C' or GST-arfaptin2 and subjected to western blotting or immunostaining; they recognized neither recombinant nor endogenous proteins. These antiserums specifically detected there antigens.

Expression of Arl1(d17N,Q71L) disperses golgin245 and arfaptin2

Arl1 enriches on the trans side of the Golgi (Hong et al., 2001) and seems to colocalize with golgin245 and arfaptin2. COS-b cells were transfected with constructs expressing various mutant form of Arl1 tagged at its C-terminus with a myc tag then subjected to indirect immunofluorescence staining. Ecotopic expressed Arl1 was targeted to Golgi marked by p58 or βCOP and didn't affect the staining pattern of endogenous golgin245 or arfaptin2. Constitutively inactive mutant of Arl1 , Arl1(T31N) generated by substituting Thr at position 31 with Asn are regarded as a dominant –negative form and disassemble Golgi apparatus but not Golgi matrix (Hong et al., 2001). But Arl1(T31N) didn't change Golgi-localization of golgin245, p58 and βCOP. Expression of Arl1(Q71L) a preferencially GTP-bound form causes engorgement of Golgi (Kahn et al., 2001) and arrests the transport of VSV-G from Golgi to cell surface (Hong et al., 2001). The active form of small GTPase are predicted to interact with its effectors and more resistant to BFA treatment. However overexpression of Arl1(Q71L) decreased golgin245-labeling signal; one interpretation could be that a part of epitopes of golgin245 were masked because of interacting with Arl1(Q71L). It's interesting to verify whether the active form of Arl1 influence golgin245. Arl1(d17N, Q71L) created by deleting the N-terminal residues 1-17 of Arl1(Q71L) dispersed evenly in nucleoplasm and cytoplasm when expressed in COS-b cells. Perinuclear Golgi localization of golgin245 but not of p58 and β-COP was disrupted in cells expressing Arl1(d17N, Q71L). Therefore,

Arl1(d17N, Q71L) specifically affects the trans-Golgi structure and doesn't change the cis-Golgi localization of p58 and β-COP. To specify the effect of Arl1(d17N, Q71L), Arl1(G2A) was produced by replacing Gly at position 2 with Ala of Arl1 and it was detected in the cytosol and nucleoplasm without Golgi enrichment. Although Arl1(G2A) and Arl1(d17N, Q71L) had the same distribution, Arl1(G2A) totally didn't scatter golgin245. It should be addressed that according to Hong et al.'s reporting, VSV-G transport wasn't abolished in cells overexpressing Arl1(G2A, Q71L) displayed as Arl1(G2A). On the other hand, Golgi association of Imh1p a yeast protein containing GRIP domain doesn't dependent on yeast Arl1 ( ) and the GRIP domain is considered binding to a Golgi-specific lipid instead of proteins (Nichols et al., 1999). Because of its high molecular weight, golgin245 is proposed to be a trans-Golgi scaffold protein. It seems like that the constitutively active form of Arl1 modulates the function of golgin245 by binding to the C-terminal region of golgin245.

Based on the same approach, we found that arfaptin2 was scattered from Golgi only in cells expressing Arl1(d17N, Q71L). Arfaptin2 has been reported to mediate cross-talk between Rac and ARF signaling pathway (Smerdon et al., 2001; Exton et al., 2001) and interact with Arl1 to increase GTPγS binding (Kahn et al., 2001). There are 60% identity and 81% homology between arfaptin1 and 2; by using in vitro translocation study, arfaptin1 in HL-60 cell cytosol is recruited by ARF to Golgi membrane in a GTPγS-dependent and brefeldin A-sensitive manner (Exton et al., 1997). In addition, arfaptin1 is a potent inhibitor of ARF actions on cholera toxin and phospohlipase D (Kanoh et al., 1998). It's important to demonstrate how the function of Arl1 is modified by arfaptin2.

Arl1 interacts with golgin245 and arfaptn2 in vivo

It's important to analysis whether Arl1 affects golgin245 directly or not. At first, a construct containing the C-termial residues 2033-2229 having GRIP domain of golgin245 was inframe fused with a Flag tag at N terminus, termed Flag-golgin245C'dN. 293T cells were transfected with Flag-golgin245C'dN and different forms of Arl1-TAP and subjected to pull down. All of the Arl1-TAP mutants and TAP protein were precipitated by IgG beads, but only Arl1(Q71L)-TAP and Arl1(d17N, Q71L)-TAP could precipitate Flag-golgin245C'dN. To confirm that, endogenous golgin245 in 293T cell lysate was precipitated by anti-golgin245 antibody and endogenous Arl1 was coprecipitated. This result supports the hypothesis that activated Arl1 modifies the function of golgin245 by associating with it.

GDP-bound form of Arl1 colocalizes with lysozome

Arl1(T31N) displayed a punctate distribution around Golgi and the pattern looked like vesicles transported between Golgi and plasma membrane. After cells were transfected with Arl1(T31N)-myc then processed to double labeling with anti-myc antibody and lysotracker. The signal of Arl1(T31N) and lysotracker partially overlapped. To investigate whether Arl1(T31N) is resident in lysosome; we examined the distribution of LAMP-1 a lysosome protein and EEA1 a endosome protein in cells expressing Arl1(T31N).

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