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The KDEL Receptor Regulates a GTPase-activating Protein for ADP-ribosylation Factor 1 by Interacting with Its Non-catalytic Domain*

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ADP-ribosylation factor 1 (ARF1) is a key regulator of transport in the secretory system. Like all small GTPases, deactivation of ARF1 requires a GTPase-activating protein (GAP) that promotes hydrolysis of GTP to GDP on ARF1. Structure-function analysis of a GAP for ARF1 revealed that its activity *in vivo* requires not only a domain that catalyzes hydrolysis of GTP on ARF1 but also a non-catalytic domain. In this study, we show that the non-catalytic domain of GAP is required for its recruitment from cytosol to membranes and that this domain mediates the interaction of GAP with the transmembrane KDEL receptor. Blocking its interaction with the KDEL receptor leaves the GAP cytosolic and prevents the deactivation *in vivo* of Golgi-localized ARF1. Thus, these findings suggest that the KDEL receptor plays a critical role in the function of GAP by regulating its recruitment from cytosol to membranes, where it can then act on its membrane-restricted target, the GTP-bound form of ARF1.

Transport among intracellular membrane compartments is accomplished by membrane-bound carriers that are formed by the recruitment of cytosolic coat proteins onto membranes. Upon delivery to a target compartment, coat proteins must be released to the cytosol before transport carriers can fuse with the compartment. Members of the ADP-ribosylation factor (ARF)[1] family of small GTPases regulate the recruitment of coat proteins. Binding of GTP activates ARF1 and stabilizes its association with target membranes. In the early secretory system, the stabilized association of ARF1 with Golgi membranes leads to the recruitment of the cytosolic COPI coat proteins. Subsequently, hydrolysis of its bound GTP to GDP deactivates ARF1. As a result, both ARF1 and COPI are released from membranes to cytosol (1–3).

Like all small GTPases, interconversion of ARF1 between its two states requires catalysis, which is accomplished by a guanine nucleotide exchange factor (GEF) that enhances exchange of GDP for GTP and a GTPase-activating protein (GAP) that promotes hydrolysis of GTP to GDP. Several GEFs (4–7) and GAPs (8–10) for ARF1 have been identified based on their ability to catalyze *in vitro* the GTPase cycle of ARF1. However, the *in vivo* role of these regulators remains to be established in many cases, because their ability to localize to the same subcellular compartments as ARF1 and regulate its effector functions in these compartments remains uncertain.

A GAP for ARF1 has been identified and shown to localize to the Golgi complex (8). When overexpressed in mammalian cells, this ARF1 GAP induces a phenotype that is consistent with deactivation of Golgi-localized ARF1 (11). This phenotype is manifest by the release of COPI from the Golgi complex and redistribution of the entire Golgi complex to the ER. Using this phenotype as an *in vivo* assay, we have identified at least two functional domains in ARF1 GAP (12). The catalytic domain resides in the amino portion of the protein as a truncated form of GAP that contains its first 257 amino acids is fully active in the *in vitro* GAP assay. However, this truncation mutant shows reduced activity in the *in vivo* assay of ARF1 deactivation. Thus, a non-catalytic domain that includes parts of the carboxyl terminus of GAP is also required for GAP activity on ARF1 *in vivo*. Because the GTP-bound form of ARF1 is restricted to membranes (13–16) and cytosolic GAP must be recruited to membranes to act on its target, one possibility is that the non-catalytic domain of GAP may be important in mediating this recruitment.

Relevant to this possibility, we had previously shown that the transmembrane KDEL receptor associates with ARF1 GAP (11). The KDEL receptor was originally defined to recognize a large class of soluble ER proteins with a carboxyl-terminal motif of lysine-aspartate-glutamate-leucine (KDEL) (17, 18). These KDEL proteins perform essential functions in the ER related to protein folding and assembly (19). Whenever these proteins escape from the ER and reach the Golgi complex, they are retrieved to the ER by the KDEL receptor (20). The possibility that the KDEL receptor not only retrieves KDEL proteins but also regulates transport in the early secretory pathways was suggested initially by observations of yeast mutants with deleted KDEL receptors. These mutants not only could not retrieve KDEL proteins but also had dysregulated transport through the Golgi complex (17). Elucidating how the KDEL

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[1] The abbreviations used are: ARF, ADP-ribosylation factor; GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; ER, endoplasmic reticulum; HA, hemagglutinin; GFP, green fluorescent protein; BFA, brefeldin A; COP, coat protein.

receptor regulates transport, we showed that overexpression of the KDEL receptor induces a phenotype of ARF1 deactivation by interacting with ARF1 GAP (11). Moreover, ligand binding by the KDEL receptor regulates its interaction with GAP (21). Thus, regulation of transport through the KDEL receptor appears fundamentally similar to many signal transduction processes in which membrane receptors act through either a GAP or GEF of key small GTPases to regulate different cellular events (22).

Although its interaction with ARF1 GAP is necessary for the KDEL receptor to affect ARF1 (11), it remains unclear whether GAP might also require this interaction to act on ARF1 *in vivo*. In this study, we find that the non-catalytic domain of GAP, which is essential for GAP activity *in vivo*, mediates the recruitment of cytosolic GAP to membranes and its interaction with the KDEL receptor. Abrogating this interaction redistributes GAP to the cytosol, where it no longer exhibits activity on ARF1 *in vivo*. Thus, interaction with the KDEL receptor is critical for GAP to act on ARF1 *in vivo*.

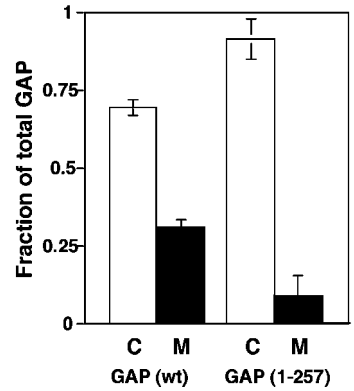

FIG. 1. **Membrane localization of ARF1 GAP is enhanced by its non-catalytic domain.** HeLa cells were transfected with construct encoding either wild type (*wt*) rat GAP or mutant rat GAP-(1–257) and fractionated into total membranes (*M*) and cytosol (*C*). Equivalent fractions of membranes and cytosol were then analyzed for ARF1 GAP by immunoblotting. Results from three separate experiments were quantified and then calculated for mean and standard error.

EXPERIMENTAL PROCEDURES

Cells and Antibodies—HeLa and COS-7 cells were grown in complete medium that consisted of Dulbecco's modified essential medium (Life Technologies, Inc.) with 10% fetal calf serum, 2 mM glutamine, and 40 μg/ml gentamicin at 37 °C in a 5% CO_2 incubator. A HeLa cell line that stably expresses the Myc-tagged KDEL receptor had been generated as described previously (11).

The following antibodies were used: mouse monoclonal antibody 9E10 against the Myc epitope (ATCC, Manassas, VA), mouse monoclonal antibody against the hemagglutinin (HA) epitope (11), mouse monoclonal antibody against the 6x-His epitope (CLONTECH, Palo Alto, CA), rabbit polyclonal antiserum against ARF1 GAP (8), mouse monoclonal antibody against p64 (provided by J. Deng, Pittsburgh, PA), mouse monoclonal antibody M3A5 against β-COP (provided by T. Kreis, Geneva, Switzerland), mouse monoclonal antibody H68.4 against transferrin receptor (provided by I. Trowbridge, La Jolla, CA), and mouse monoclonal antibody AF8 against calnexin (provided by M. Brenner, Boston, MA). Fluorescein-conjugated donkey antibody against mouse IgG, fluorescein-conjugated donkey antibody against rabbit IgG, rhodamine-conjugated donkey antibody against mouse IgM, indocarbocyanine-conjugated donkey antibody against mouse IgG, and indocarbocyanine-conjugated donkey antibody against rabbit IgG were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA).

Plasmids and Transfection—The following cDNAs were used and have been described previously: Myc-tagged wild type KDEL receptor (23), HA-tagged wild type KDEL receptor and mutant receptor 5TM (11), HA-tagged wild type ARF6 (24), and 6x-His-tagged wild type GAP and mutant GAP1-(1–257) (12). To generate a cell line that stably expressed wild type ARF1 fused with green fluorescent protein (GFP), HeLa cells were transfected with a cDNA that encoded ARF1-GFP (cloned into the pEGFPN1 vector from CLONTECH) using LipofectAMINE (Life Technologies, Inc.). Transfected cells were then selected with 800 μg/ml G-418 (Life Technologies, Inc.), sorted by flow cytometry for maximal expression of ARF1-GFP, and maintained in Dulbecco's modified essential medium with 10% fetal bovine serum and 150 μg/ml G-418.

Microscopy and Biochemical Studies—Immunofluorescence microscopy, immunoprecipitation, immunoblotting, and subcellular fractionation were performed as described previously (11).

Assay for Recruitment of Cytosolic GAP to Membranes—HeLa cells (approximately 5×10^8) were scraped and washed with phosphate-buffered saline twice and with homogenization buffer (10 mM triethanolamine, pH 7.4, 250 mM sucrose, 1 mM EDTA) once. The cell pellet was resuspended in four volumes of homogenization buffer and then sheared by four passes through a ball-bearing homogenizer at 36 μm clearance (EMBL machine shop, Heidelberg, Germany). Nuclei and cell debris were removed by centrifugation at $500 \times g$ for 10 min. The postnuclear supernatant was subjected to ultracentrifugation for 2.5 h at $200{,}000 \times g$ through a sucrose step gradient of 20, 30, and 70% (w/v) sucrose in 10 mM triethanolamine, pH 7.4, and 1 mM EDTA. Membranes were recovered from the 30–70% interface, and cytosol was recovered from the phase above 20% sucrose. Both fractions were adjusted to 2.5 mM $MgCl_2$ final concentration and stored at −80 °C.

For the GAP recruitment assay, 1/20 of each fraction was used for individual incubation conditions, in which they were incubated together at 37 °C for 15 min followed by centrifugation at $14{,}000 \times g$ for 10 min. Equivalent fractions of the pellet and the supernatant were then subjected to SDS-polyacrylamide gel electrophoresis followed by immunoblotting using antibodies against β-COP, GAP, and calnexin. Quantitation of immunoblots was performed by ImageQuant (Molecular Dynamics, Sunnyvale, CA).

Kinetics of ARF1 Dissociation from the Golgi Complex in the Presence of Brefeldin A (BFA)—HeLa cells stably expressing ARF1-GFP were generated and then transiently transfected with the mutant KDEL receptor 5TM using LipofectAMINE in serum-free Dulbecco's modified essential medium (Life Technologies, Inc.). Between 24 and 48 h after transfection, the kinetics of ARF1-GFP dissociation from the Golgi complex was measured as described previously (25). Briefly, cells on coverglass were placed in a live cell chamber and imaged using a Molecular Dynamics 2001 confocal laser-scanning microscope. Cells were bathed in phenol red-free medium during the entire experiment and were excited at 488 nm with a krypton-argon laser (3% of maximal intensity). Emitted light was passed through a 530 nm barrier filter and detected using a photomultiplier tube. Time lapse experiments were carried out by scanning cells once every 30 s. Brefeldin A was added at 5 μg/ml final concentration after the first scan. Scans of the fluorescence intensity on the Golgi complex were quantified using ImageSpace software (Molecular Dynamics). These data were further analyzed using GraphPad Prism to determine the time course of ARF1 release from the Golgi complex. In cells transiently transfected with 5TM, half-lives of ARF1-GFP on the Golgi complex fell into a bimodal distribution with one population having a half-life similar to control cells. This population represented untransfected cells, whereas the other population represented cells transfected with 5TM. These two populations were confirmed by immunofluorescence microscopy of fixed cells with double labeling for HA-tagged 5TM and ARF1-GFP. The half-lives of ARF1 dissociation from the Golgi complex were determined for both populations, grouped, and then analyzed by analysis of variance followed by Bonferroni post-test comparisons.

RESULTS

To examine the role of the non-catalytic domain in ARF1 GAP, we first compared the intracellular distribution of wild type GAP with that found in a truncated form (GAP1-(1–257)). This mutant had been used in a previous study (12) to define a non-catalytic domain in GAP because it was active *in vitro* in catalyzing hydrolysis of GTP on ARF1 but its overexpression was ineffective in promoting a phenotype of ARF1 deactivation *in vivo*. Transfecting either form of GAP into cells followed by subcellular fractionation, we found that wild type GAP associated with the membrane fraction more efficiently than did GAP-(1–257) (Fig. 1). The association of GAP-(1–257) with the membrane fraction could be attributed to its affinity to certain diacylglycerol moieties that are likely to exist on Golgi membranes (26). However, because wild type GAP associated with

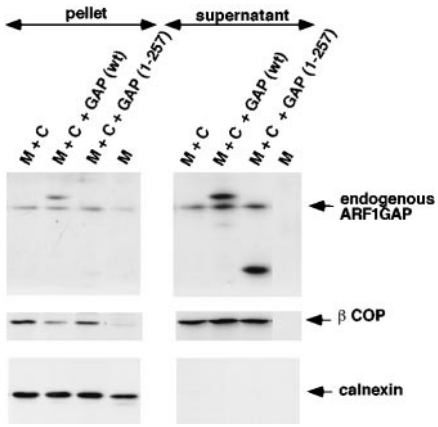

FIG. 2. **Recruitment of GAP from cytosol to membranes requires a non-catalytic domain.** Cytosol (*C*) and total membranes (*M*) derived from HeLa cells were incubated together (*M* + *C*) or were supplemented with cytosol that overexpressed wild type rat GAP (*M* + *C* + *GAP* (*wt*)) or mutant rat GAP-(1–257) (*M* + *C* + *GAP* (*1-257*)). Incubations were then centrifuged, and equivalent fractions of pellet and supernatant were analyzed by immunoblotting for ARF1 GAP (*top panel*), β-COP (*middle panel*), or calnexin (*bottom panel*; to show that membranes were quantitatively collected in pellet fractions). Note that the transfected wild type rat GAP could be distinguished from the endogenous human GAP in HeLa cells because of a slightly greater apparent molecular size (11).

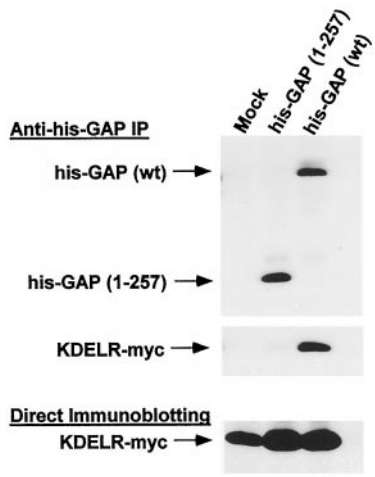

FIG. 3. **The non-catalytic domain of GAP is required for its interaction with the KDEL receptor.** A HeLa cell line stably transfected with Myc-tagged KDEL receptor was transiently transfected with vector (*Mock* condition) or with 6x-His-tagged GAP constructs (either wild type (*wt*) or truncation mutant-(1–257)). Co-precipitation was performed by immunoprecipitating for the transfected tagged GAP using an anti-6x-His antibody followed by immunoblotting for the tagged KDEL receptor using an anti-Myc antibody (*middle panel*). Immunoblotting of the same precipitates with an anti-ARF1 GAP antiserum revealed similar levels of GAP proteins precipitated (*top panel*). Direct immunoblotting of cell lysates revealed similar levels of the tagged KDEL receptor available for co-precipitation (*bottom panel*).

the membrane fraction even more efficiently, this result suggested that the non-catalytic domain of GAP potentially played an important role *in vivo* by affecting the distribution of GAP between the membrane and cytosol.

To test for this possibility, we performed an assay comparing the recruitment of wild type GAP and GAP-(1–257). For this purpose, total membranes and cytosol prepared from human HeLa cells were incubated together and supplemented with cytosol that overexpressed either wild type GAP or GAP-(1–257). Whereas a significant fraction of wild type GAP was recruited from cytosol to membranes, GAP-(1–257) remained cytosolic (Fig. 2). To examine the functional consequence of this difference in GAP recruitment, we determined the distribution of COPI between membranes and cytosol in the same experiment, because release of COPI from membranes reflects deactivation of ARF1 (27, 28). As assessed by β-COP, incubation with cytosol that contained wild type GAP resulted in less membrane-bound β-COP as compared with incubation with cytosol that contained GAP-(1–257) (Fig. 2). Thus, GAP activity on ARF1 correlated with the recruitment of cytosolic GAP to membranes, and this recruitment required the non-catalytic domain.

Because the KDEL receptor is a transmembrane protein that had been shown previously to interact with GAP (11), we next tested whether this interaction is mediated by the non-catalytic domain of GAP. When a co-precipitation study was performed by immunoprecipitating for 6x-His-tagged GAP followed by immunoblotting for the Myc-tagged KDEL receptor, a significant amount of KDEL receptor was co-precipitated with wild type GAP but not with GAP-(1–257) (Fig. 3). Thus, this result suggested that an interaction between the KDEL receptor and GAP requires the non-catalytic domain of GAP.

If the interaction with KDEL receptor were required for the recruitment of GAP to membranes, then disrupting the interaction would be predicted to prevent membrane localization of GAP. By deleting the cytoplasmic tail and the last two transmembrane domains of the KDEL receptor, we had previously generated a mutant KDEL receptor (5TM). Overexpression of 5TM blocked the interaction of the KDEL receptor with GAP by sequestering wild type KDEL receptors into oligomers that could no longer interact with GAP (11). To test whether overexpression of 5TM would prevent membrane localization of GAP, we transfected GAP either with or without 5TM into HeLa cells. Upon fractionation into total membranes and cytosol, we found that membrane distribution of GAP was significantly impaired in cells that co-overexpressed 5TM (Fig. 4).

To examine the *in vivo* consequences of preventing GAP from localizing to membranes, we assessed whether 5TM overexpression blocked the ability of overexpressed GAP to induce a phenotype of ARF1 deactivation. A manifestation of this phenotype is the redistribution of the entire Golgi complex to the ER (11, 23). Thus, we assayed by indirect immunofluorescence microscopy the integrity of the Golgi complex. Cells that overexpressed GAP alone had the Golgi complex redistributed to the ER, whereas cells that co-overexpressed 5TM and GAP no longer had the Golgi complex redistributed to the ER. (Fig. 5).

To determine whether 5TM overexpression also blocked the activity of endogenous GAP on ARF1, we examined the effect of 5TM overexpression on the distribution of ARF1 between the Golgi complex and cytosol, as this distribution reflects whether ARF1 is in its activated or deactivated form (13–16). For this purpose, we examined a fusion protein generated by attaching the GFP to ARF1. Regulation of this fusion protein has been shown to resemble that of wild type ARF1 (25). In cells with 5TM overexpression, the fluorescent signal of GFP-tagged ARF1 at the Golgi complex was more intense than in control cells (Fig. 6*A*). By quantitative confocal microscopy, this increase was approximately 2-fold, suggesting that 5TM overexpression enhanced activation of ARF1.

In principle, because the steady-state distribution of ARF1 on Golgi membranes reflects the net activities of its GAP and GEF (13, 15), 5TM overexpression could have enhanced activation of ARF1 by either enhancing its GEF activity or inhibiting its GAP activity. Thus, we examined the effect of 5TM overexpression on ARF1 localization to the Golgi complex in the presence of BFA, which blocks the contribution of GEF activity on ARF1 (16, 29, 30). Upon the addition of BFA, the rate at which ARF1 was released from the Golgi complex was

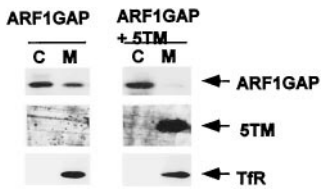

FIG. 4. **Membrane localization of GAP is markedly reduced by the overexpression of the mutant KDEL receptor 5TM.** HeLa cells were transfected with the rat form of wild type GAP either alone or in combination with HA-tagged 5TM. Transfected cells were then homogenized, fractionated into total membranes (*M*) and cytosol (*C*), and immunoblotted for GAP (*top panel*) or HA-tagged 5TM (*middle panel*). Immunoblotting for transferrin receptor (*TfR*) revealed that total membranes were quantitatively collected (*bottom panel*).

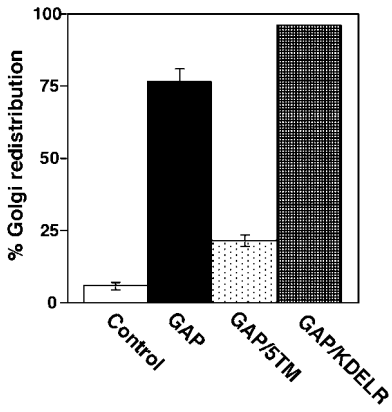

FIG. 5. **A phenotype of ARF1 deactivation induced by GAP overexpression is inhibited by the co-overexpression of the mutant KDEL receptor 5TM.** HeLa cells were transiently transfected in a mock fashion (*Control*) or with constructs encoding for GAP in combination with an expression control protein (HA-tagged ARF6), wild type KDEL receptor (*KDELR*), or mutant receptor 5TM. A phenotype of ARF1 deactivation was assessed by Golgi redistribution to the ER. Specifically, cells were evaluated by double-labeling immunofluorescence microscopy using a rabbit antibody against GAP and a mouse antibody against a Golgi marker, p64 (40). One hundred cells with overexpressed GAP were identified and assessed as to whether the Golgi marker remained intact. This procedure was performed in three separate experiments and calculated for the mean with standard error.

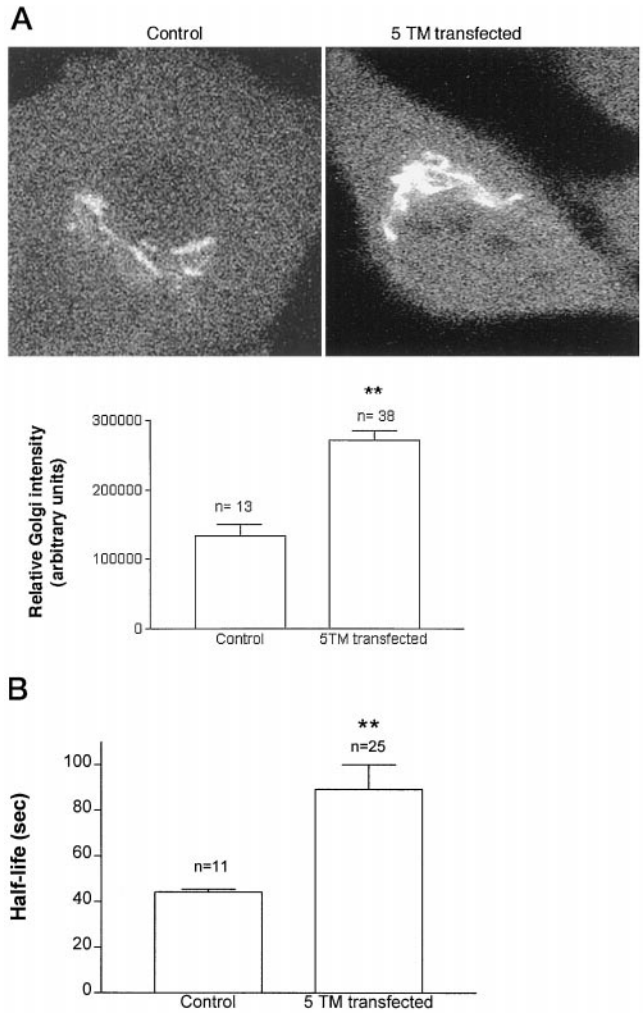

FIG. 6. **Inhibition of an interaction between GAP and the KDEL receptor reduces endogenous GAP activity on ARF1.** *A*, 5TM overexpression induces more ARF1 on the Golgi complex. A HeLa cell line stably expressing GFP-tagged ARF1 was transiently transfected with either a mock construct or a construct encoding HA-tagged 5TM. ARF1-GFP on the Golgi complex was then compared in cells that either did or did not overexpress 5TM (*upper*). The level of Golgi-localized ARF1-GFP was quantified (*lower*) for several Golgi complexes (number indicated by *n*) and calculated for the mean with standard error. *B*, 5TM overexpression decreases the rate at which ARF1-GFP detaches from the Golgi complex in the presence of BFA. A HeLa cell line stably expressing ARF1-GFP was transiently transfected with a mock construct or a construct encoding 5TM. The rate at which ARF1-GFP disappeared from the Golgi complex following BFA treatment was measured, and half-lives corresponding to those in 5TM expressing cells were grouped and compared with those in mock-transfected cells (*Control*). Three separate experiments were done to calculate a mean with standard error.

slowed by 2-fold in cells that had been transfected with 5TM as compared with the rate of ARF1 release in cells that had not been transfected with 5TM (Fig. 6*B*). This result correlated with a 2-fold increase in the steady-state distribution of ARF1 at the Golgi complex seen upon 5TM overexpression (Fig. 6*A*). Thus, collectively, the effects of 5TM overexpression suggested that GAP activity on ARF1 *in vivo* was reduced when an interaction between the KDEL receptor and GAP was disrupted.

DISCUSSION

In this study, we find that recruitment of ARF1 GAP from cytosol to membranes is mediated by its non-catalytic domain, which interacts with the transmembrane KDEL receptor. When this interaction is abrogated by a mutant KDEL receptor (5TM), GAP remains cytosolic and no longer exhibits *in vivo* activity on ARF1. Collectively, these findings suggest that the KDEL receptor plays a critical role in the activity of GAP by regulating its recruitment from cytosol to membranes, where it can then act on its membrane-restricted target, the GTP-bound form of ARF1.

Significantly, like COPI and ARF1, the GAP that regulates these key transport components is also regulated by its recruitment from cytosol to membranes. Of particular relevance to this comparison is that recruitment of COPI appears to involve interactions with both the lipid and protein components of its target membrane, as evidence exists for COPI interacting with phosphoinositide moieties of membranes (31) and also with cytoplasmic motifs of transmembrane proteins (32, 33). In this regard, GAP-(1–257) has been shown previously to interact with certain diacylglycerol moieties of membranes (26), and this may be the basis for some of its membrane association seen upon subcellular fractionation in this study. However, by itself, this lipid interaction does not seem sufficient to confer GAP activity *in vivo*, because overexpression of GAP-(1–257) cannot effectively induce a phenotype of ARF1 deactivation (12). Thus, like COPI (34), the interaction of GAP with a protein component on the target membrane is also critical for its function, and this requirement appears to be fulfilled by an interaction between the non-catalytic domain of GAP and the transmem-

brane KDEL receptor.

As the KDEL receptor cycles between the ER and the Golgi complex, regulating GAP recruitment through the KDEL receptor would seemingly localize GAP equally well to membranes of both the ER and the Golgi complex. However, GAP is localized mostly to the Golgi complex (8). An explanation is suggested by our previous observation that ligand binding by the KDEL receptor regulates its association with GAP (21). Thus, because KDEL proteins bind to the KDEL receptor at the Golgi complex (20), GAP would be recruited mainly to Golgi membranes. However, as ARF1 deactivation by its GAP promotes the release of COPI from its target membranes (27, 28), activation of GAP by the KDEL receptor at the Golgi complex would seemingly prevent the recruitment of COPI onto Golgi membranes. Yet, ligand binding by the KDEL receptor induces its movement into retrograde COPI-coated vesicles at the Golgi complex (35). Thus, as the yeast homologues of GAP has been shown to regulate retrograde transport from the Golgi complex to the ER (36), another possibility is that GAP is being recruited to Golgi membranes by the KDEL receptor to regulate transport mediated by retrograde COPI-coated vesicles.

In considering how these two apparently incongruous possibilities can be reconciled, a potential insight is that the target of GAP is the activated form of ARF1 (8). Thus, rather than competing with the GEF that activates ARF1, and thereby preventing the recruitment of COPI onto its target membranes altogether, the GAP recruited to Golgi membranes by the KDEL receptor may act only after GEF has acted on ARF1 to initiate the formation of COPI-coated vesicles. Relevant to this consideration, COPI has recently been shown to regulate the catalytic activity of GAP on ARF1 (37). A possibility based on this observation is that, as more COPI is being recruited to form coated buds, GAP may be activated to release ARF1 from membranes of newly forming vesicles. Thus, rather than acting on ARF1 after the complete formation of COPI-coated vesicles, GAP might act during a late stage of vesicle maturation. This scenario would be similar to findings on transport mediated by COPII (38) or clathrin AP-1 (39), where their mature coated vesicles were found to lack a significant level of the responsible small GTPase, suggesting that the relevant GAP acts during the maturation of these vesicles. Thus, future elucidation of how the interaction between the KDEL receptor and GAP regulates retrograde transport will likely contribute to determining the precise role of GAP in retrograde transport from the Golgi complex to the ER.

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The KDEL Receptor Regulates a GTPase-activating Protein for ADP-ribosylation Factor 1 by Interacting with Its Non-catalytic Domain

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