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The KDEL receptor, ERD2, regulates intracellular traffic by recruiting a GTPase-activating protein for ARF1

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The small GTPase ADP-ribosylation factor 1 (ARF1) is a key regulator of intracellular membrane traffic. Regulators of ARF1, its GTPase-activating protein (GAP) and its guanine nucleotide exchange factor have been identified recently. However, it remains uncertain whether these regulators drive the GTPase cycle of ARF1 autonomously or whether their activities can be regulated by other proteins. Here, we demonstrate that the intracellular KDEL receptor, ERD2, self-oligomerizes and interacts with ARF1 GAP, and thereby regulates the recruitment of cytosolic ARF1 GAP to membranes. Because ERD2 overexpression enhances the recruitment of GAP to membranes and results in a phenotype that reflects ARF1 inactivation, our findings suggest that ERD2 regulates ARF1 GAP, and thus regulates ARF1-mediated transport.
Keywords: ARF1/GTPase-activating protein/intracellular transport/KDEL receptor/signal transduction

Introduction

Intracellular transport is accomplished by the regulated formation of membrane-bound carriers that shuttle between the different intracellular membrane compartments of the secretory pathway (Rothman and Wieland, 1996; Schekman and Orci, 1996). Studies of the endoplasmic reticulum (ER) and the Golgi complex, that have provided much of our current understanding of how intracellular transport is regulated, suggest that a cytosolic coat protein complex, COPI, attaches to membranes to initiate the formation of transport vesicles (Orci *et al.*, 1989). These coated vesicles then target to another compartment where COPI must be released to the cytosol before the vesicles can fuse with their target compartment (Orci *et al.*, 1989).

Recruitment of COPI to membranes is regulated by a small GTPase named ADP-ribosylation factor 1 (ARF1). Activation of ARF1 by its binding of GTP (ARF1-GTP) recruits COPI from the cytosol to membranes (Donaldson *et al.*, 1992; Palmer *et al.*, 1993), while ARF1 inactivation by the hydrolysis of GTP to GDP bound to ARF1 (ARF1-GDP) results in the release of COPI from membranes back to the cytosol (Tanigawa *et al.*, 1993). Like all small GTPases, the GTPase cycle of ARF1 requires a guanine nucleotide exchange factor (GEF) (Chardin *et al.*, 1996; Morinaga *et al.*, 1996; Meacci *et al.*, 1997) to catalyze ARF1 activation and a GTPase-activating protein (GAP) to catalyze ARF1 inactivation (Cukierman *et al.*, 1995). The GAP and GEF of ARF1 have been identified recently. However, it remains unclear whether they function autonomously in catalyzing the GTPase cycle of ARF1 or whether other proteins may regulate their catalytic activities.

We became interested in identifying potential regulatory proteins during studies of an intracellular, multi-spanning membrane receptor named ERD2 (Pelham, 1991). ERD2 recognizes a C-terminal motif of lysine–aspartate–glutamate–leucine (KDEL) on certain soluble ER proteins (Lewis *et al.*, 1990; Wilson *et al.*, 1993) and retrieves those proteins that have leaked to the Golgi complex back to the ER (Lewis and Pelham, 1992). The KDEL-containing proteins, dubbed KDEL proteins, are abundant and ubiquitous. They perform essential functions in the ER related to protein folding and assembly, such as the immunoglobulin-binding protein (BiP) that associates with intermediate states of folded proteins and helps in their proper folding (Pelham, 1989). ERD2 was identified originally in studies of mutant yeast cells that failed to retrieve KDEL proteins, and hence the name of ER retention-defective complementation group 2 (Semenza *et al.*, 1990). There are two human homologs of ERD2 that appear to function similarly (Lewis and Pelham, 1990; Hsu *et al.*, 1992).

A clue that ERD2 may also function to regulate transport came from observations of mutant yeast cells with deleted ERD2. These cells had profoundly enlarged and disorganized Golgi complexes with dysregulated transport in the early secretory system (Semenza *et al.*, 1990). Accordingly, the overexpression of the human homologs of ERD2 also profoundly affected transport and organelle structure, but in a fashion reminiscent of perturbations that inhibited ARF1 GEF activity (Hsu *et al.*, 1992). This finding pointed to an intriguing possibility that ERD2 affected transport through ARF1.

Here, we demonstrate that ERD2 oligomerizes and interacts with ARF1 GAP, events that result in the recruitment of cytosolic GAP to membranes and ARF1 inactivation. Thus, our findings suggest that ERD2 regulates ARF1-mediated transport through its interaction with ARF1 GAP.

Results

A phenotype reflecting ARF1 inactivation

In principle, one can determine whether ERD2 overexpression affects the GTPase cycle of ARF1 by isolating ARF1

from cells that overexpress ERD2 and assessing the ratio of GTP/GDP bound to ARF1. However, such a direct approach cannot measure the level of ARF1-GTP accurately *in vivo*, because binding of GTP to ARF1 is unstable (Weiss *et al.*, 1989; Franco *et al.*, 1995). In addition, the high ARF1 GAP activity present on membranes prevents the possibility of using subcellular fractionation to assess the membrane-bound fraction of ARF1 as a reflection of ARF1-GTP (Helms *et al.*, 1993; Ostermann *et al.*, 1993). Studies of other small GTPases have circumvented this problem by using a phenotype that specifically reflects altered effector functions due to the exaggeration of either an activated (GTP-bound) or inactivated (GDP-bound) state of the small GTPase (Lowy and Willumsen, 1993; Machesky and Hall, 1996).

A phenotype induced either pharmacologically with a drug named brefeldin-A (BFA) (Doms *et al.*, 1989; Lippincott-Schwartz *et al.*, 1989; Donaldson *et al.*, 1990) or by the overexpression of an ARF1 mutant (T31N) (Dascher and Balch, 1994; Peters *et al.*, 1995) has been attributed to an inhibition of ARF1 GEF. Thus, preventing ARF1 activation maintains COPI in the cytosol, and the net result of maintaining Golgi membranes uncoated is that they undergo uncontrolled homotypic membrane fusion with the ER to form a fused compartment that is incapable of normal transport (Klausner *et al.*, 1992; Elazar *et al.*, 1994). To validate that this phenotype specifically reflects ARF1 inactivation, we took advantage of the fact that inactivating ARF1 by inhibiting its GEF can also be accomplished by enhancing its GAP (Donaldson and Klausner, 1994). Thus, we examined whether enhancing GAP activity by the overexpression of GAP would result in a similar phenotype.

ARF1 GAP (Cukierman *et al.*, 1995) was transiently transfected into cells, and its effects were examined by immunofluorescence microscopy. Upon GAP overexpression, the distribution of COPI (as detected through one of its subunits, β-COP) changed from a predominantly tight, juxtanuclear distribution to a completely diffuse, hazy distribution, suggesting that COPI had been released from intracellular membranes (including that of the Golgi complex) to the cytosol (Figure 1A). Concurrently, the localization of a Golgi marker changed from a tight, juxtanuclear distribution to a diffuse, reticular distribution (Figure 1B), suggesting that the Golgi complex was redistributed to the ER. To assess general secretion, lysozyme was used as a reporter protein to assess secretion in a metabolic pulse–chase experiment. While lysozyme was normally secreted efficiently, its secretion was blocked by GAP overexpression (Figure 1C). Thus, GAP overexpression induced a phenotype similar to that induced by an inhibition of ARF1 GEF activity, suggesting that this phenotype can be used as an *in vivo* readout for ARF1 inactivation.

In support of this interpretation, we asked whether ARF1 overexpression, which has been demonstrated previously to supply more activated ARF1 (Peters *et al.*, 1995), could reverse the phenotype attributed to ARF1 inactivation. As Golgi redistribution to the ER is the most specific manifestation of this phenotype, we used a previously developed biochemical assay to monitor the activation status of ARF1. Normally, glycoproteins that transit through the medial Golgi complex acquire complex

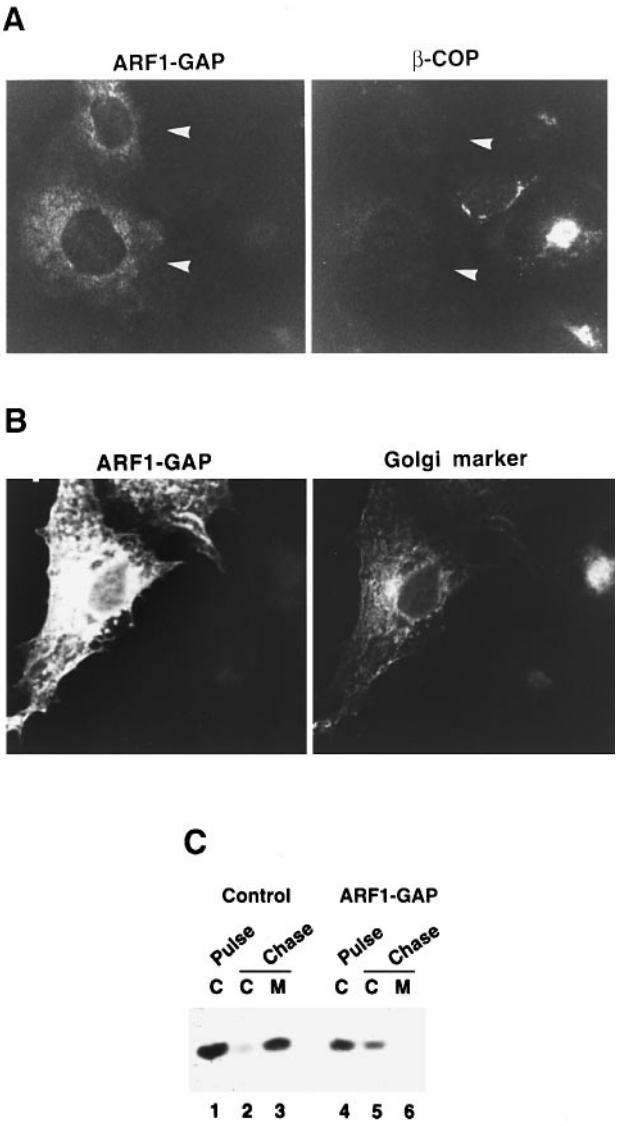

Fig. 1. GAP overexpression results in a phenotype similar to that induced by an inhibition of ARF1 GEF activity. (**A**) β-COP is changed from an intracellular membrane distribution that includes the Golgi complex to a cytosolic-like distribution. COS cells were transiently transfected with GAP (arrowhead), and then evaluated by immunofluorescence microscopy with double labeling using rabbit anti-ARF1 GAP antiserum (Cukierman *et al.*, 1995) and mouse anti-β-COP antibody (M3A5) (Allan and Kreis, 1986). (**B**) The Golgi complex is redistributed to the ER. COS cells were transiently transfected with GAP, and then evaluated by immunofluorescence microscopy with double labeling using rabbit anti-ARF1 GAP and *Lens culinaris* lectin (Hsu *et al.*, 1992). (**C**) Secretion is blocked. HeLa cells were transiently transfected with lysozyme and either control construct (lanes 1, 2 and 3) or GAP (lanes 4, 5 and 6), and assessed for lysozyme secretion; cells (C), media (M).

glycosylation that renders them resistant to endoglycosidase H (endo H). However, an ER glycoprotein that does not reach the medial Golgi complex would remain sensitive to endo H, unless the Golgi complex (with its resident glycosidases) is redistributed to the ER. Thus, to detect Golgi redistribution to the ER, we assessed the acquisition of endo H resistance of an ER glycoprotein, Tac-E19 (Hsu *et al.*, 1992). Tac-E19 acquired endo H resistance upon overexpression of the ARF1-T31N mutant, GAP or ERD2 (Figure 2). Significantly, the degree of endo H resistance

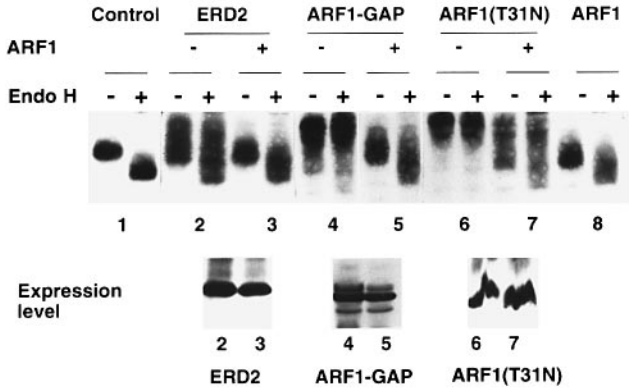

Fig. 2. ARF1 overexpression reverses the phenotype of ARF1 inactivation. HeLa cells were transiently transfected with Tac-E19 in combination with control construct (lane 1), ERD2-HA and control construct (lane 2), ERD2-HA and ARF1 (lane 3), GAP and control construct (lane 4), GAP and ARF1 (lane 5), ARF1(T31N)-HA and control construct (lane 6), ARF1(T31N)-HA and ARF1 (lane 7) or ARF1 and control construct (lane 8), and then assessed for Golgi-specific glycosylation of Tac-E19 (upper panel). Levels of ERD2-HA, GAP or ARF1(T31N)-HA either expressed alone or co-overexpressed with ARF1 were assessed by immunoblotting of cell lysates with either an anti-HA antibody or an anti-ARF1 GAP antiserum (lower panel).

was reduced by the co-overexpression of ARF1 (Figure 2). Thus, these results further supported the suggestion that a common phenotype induced by either inhibiting ARF1 GEF or enhancing ARF1 GAP was indicative of ARF1 inactivation.

Furthermore, as ERD2 overexpression resulted in a similar phenotype (Hsu *et al.*, 1992) and this phenotype was similarly reversed by the co-overexpression of ARF1 (Figure 2), these observations suggested that ERD2 overexpression also inactivated ARF1. As confirmation, we examined by immunogold electron microscopy (EM) whether the morphologic effects induced by ERD2 overexpression were reversed by ARF1 co-overexpression. Cells that co-overexpressed ERD2 and ARF1 exhibited normal-appearing Golgi complexes (Figure 3A), while cells that overexpressed only ERD2 revealed no intact Golgi complexes (Figure 3B). This difference was confirmed by stereology that quantified intact Golgi profiles (Table I). Thus, both biochemical and morphological assessments suggested that ERD2 overexpression perturbed intracellular traffic by inactivating ARF1.

ERD2 interacts with ARF1 GAP

We next examined whether overexpressed ERD2 might inactivate ARF1 through the ARF1 GAP by assessing for a potential interaction between ERD2 and GAP using a co-precipitation approach. Cells were transiently transfected with ERD2-myc and then lysed with a Triton X-100 buffer. Upon immunoprecipitation with an anti-myc antibody for ERD2-myc, followed by Western blotting and detection of GAP through an anti-GAP antibody, an interaction between ERD2-myc and GAP was detected (Figure 4A). As myc tagging did not affect the previously defined functions of ERD2 (Semenza *et al.*, 1990; Hsu *et al.*, 1992; Lewis and Pelham, 1992), we used an epitope-tagged form of ERD2, because the available antibody against ERD2 (Tang *et al.*, 1994) did not immunoprecipitate ERD2 efficiently, and tagging with different epitopes facilitated subsequent studies on the oligomerization of ERD2 (see below). As a control that epitope tagging did not contribute to the interaction between ERD2 and GAP, immunoprecipitation of a transfected myc-tagged CD8 construct did not detect its co-precipitation with GAP (Figure 4A). The interaction of ERD2 with GAP was also verified by co-precipitation through ARF1 GAP. As the existing anti-ARF1 GAP antibody did not immunoprecipitate GAP, we epitope-tagged GAP with hexameric histidine (6×his), and then transfected this construct into a cell line that expressed ERD2-myc. When immunoprecipitated for 6×his-GAP with an anti-6×his antibody, followed by immunoblotting for ERD2-myc with anti-myc antibody, an association between 6×his-GAP and ERD2-myc was detected (Figure 4B).

To gain insight into the biological significance of an interaction detected between ERD2 and GAP, we examined whether this interaction occurred under physiological conditions, when ERD2 was unable to induce a phenotype of ARF1 inactivation. We had noted previously that transient transfection of ERD2-myc resulted in its variable expression within a cell population. Only cells that markedly overexpressed ERD2-myc exhibited the phenotype of ARF1 inactivation, while cells that expressed ERD2-myc at moderate levels appeared normal (Hsu *et al.*, 1992). This observation suggested that cell lines that stably expressed moderate levels of ERD2 could be generated by selecting for those cells that grew normally, as those cells would be likely to have a growth advantage over cells that exhibited the phenotype of ARF1 inactivation. Indeed, several cell lines that stably expressed ERD2-myc were generated. Using an antibody that recognized native ERD2 (Tang *et al.*, 1994), we compared the level of ERD2 expression in cells that were transiently transfected or stably transfected with ERD2-myc. Transiently transfected ERD2-myc was expressed at at least 10-fold higher levels than endogenous ERD2 (Figure 5A). On the other hand, stably transfected ERD2-myc was expressed at levels less than endogenous ERD2 (Figure 5A).

The effects of stably transfected ERD2-myc on intracellular transport were then assessed in more detail. By immunogold EM, stably transfected ERD2-myc was distributed mainly at the Golgi complex, the intermediate compartment and the ER (Figure 5B). This intracellular distribution was similar to that previously described for endogenous ERD2 (Griffiths *et al.*, 1994). β-COP was distributed in the early secretory compartments (Figure 5C) similarly to its distribution previously described in untransfected cells (Duden *et al.*, 1991; Oprins *et al.*, 1993). Morphologically, Golgi complexes appeared as normal, distinct entities with characteristic flattened cisternae and budding rims (Figure 5A and B) and, biochemically, Tac-E19 introduced into the stable cell line remained equally as endo H sensitive as in a control cell line (Figure 5D). Thus, the stable cell line exhibited no evidence of Golgi redistribution to the ER. Finally, lysozyme secretion appeared unaffected by the stable expression of ERD2-myc (Figure 5E), suggesting that general secretion remained normal. Thus, by all criteria previously established to define the phenotype of ERD2 overexpression, cell lines stably expressing ERD2-myc exhibited no aspect of this

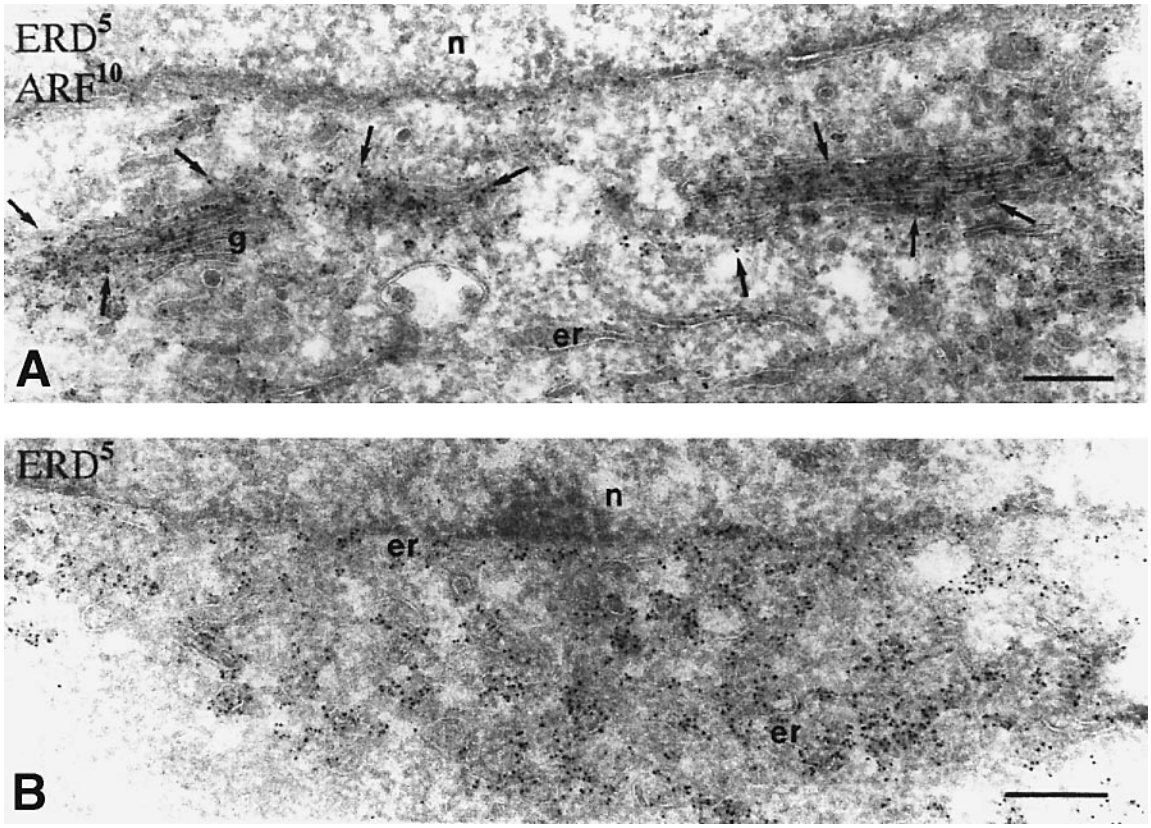

Fig. 3. Cells that co-overexpressed ERD2 and ARF1 have intact Golgi complexes. HeLa cells were transiently transfected with either both ERD2-myc and ARF1-HA (**A**) or ERD2-myc alone (**B**) and then evaluated by immunogold EM with double labeling using anti-myc (5 nm gold) and anti-HA (10 nm gold, highlighted by arrows). Bar, 200 nm; n, nucleus; er, ER; g, Golgi complex.

phenotype, suggesting that transport was normal in these cells.

In these stable transfectants, an interaction between ERD2-myc and GAP was also seen (Figure 6A, upper panel). Significantly, this interaction was enhanced upon ERD2 overexpression (Figure 6A, upper panel), as assessed by the level of GAP co-precipitated with equivalent levels of immunoprecipitated ERD2-myc from either stable transfectants or transient transfectants (Figure 6A, lower panel).

ERD2 self-oligomerizes

To examine how ERD2 overexpression induced an enhanced interaction between ERD2 and GAP, we sought clues from a conserved regulatory process common to many different cellular events. For example, growth factor receptors oligomerize and recruit effectors that mediate a signaling response to receptor activation by either ligand or receptor overexpression (Heldin, 1995). Similarly, a recently characterized signaling pathway, termed the unfolded protein response, also uses protein oligomerization to transduce a signal from the ER to the nucleus in response to stress (Shamu and Walter, 1996).

As protein oligomerization appears to be a universal mechanism for transducing an effector function, we examined whether ERD2 oligomerization might be important for its interaction with GAP. First, cell lines were generated that stably expressed both ERD2-myc and ERD2-HA at physiologic levels. These cells exhibited normal transport, as assessed by the same criteria that we had used to assess the stable transfectants expressing ERD2-myc alone (not shown). ERD2 oligomerization was detected in these stable transfectants by the co-precipitation of ERD2-myc and ERD2-HA, upon immunoprecipitation of ERD2-myc using an anti-myc antibody followed by Western blotting and detection of ERD2-HA with an anti-HA antibody (Figure 6B, upper panel). Moreover, ERD2 oligomerization was enhanced upon ERD2 overexpression (Figure 6B, upper panel), as assessed by equivalent levels of ERD2-myc immunoprecipitated from either transient or stable transfectants that express both ERD2-myc and ERD2-HA (Figure 6B, lower panel).

Table I. Co-overexpression of ARF1 restores Golgi complexes in cells with overexpressed ERD2

Cells	Cells with intact Golgi profiles	Point hits of Golgi profiles
Untransfected	20/20	13.5 ± 4.3
ERD2-myc	0/20	0.0 ± 0.0
ERD2-myc/ARF1-HA	20/20	12.9 ± 4.6

Juxtanuclear regions were randomly selected from 20 cells that were transfected as indicated. Transfected cells were chosen for similar degrees of high ERD2-myc expression (defined in Table II as 'greatly overexpressed'). Point hits of Golgi profiles refer to the number of intersections that coincided with Golgi profiles using an overlay lattice grid of 10 mm squares. This difference was insignificant ($P = 0.66$) between the double transfectants of ERD2-myc/ARF1-HA and untransfected cells, but was significant ($P < 1 \times 10^{-10}$) between the double transfectants of ERD2-myc/ARF1-HA and single transfectants of ERD2-myc.

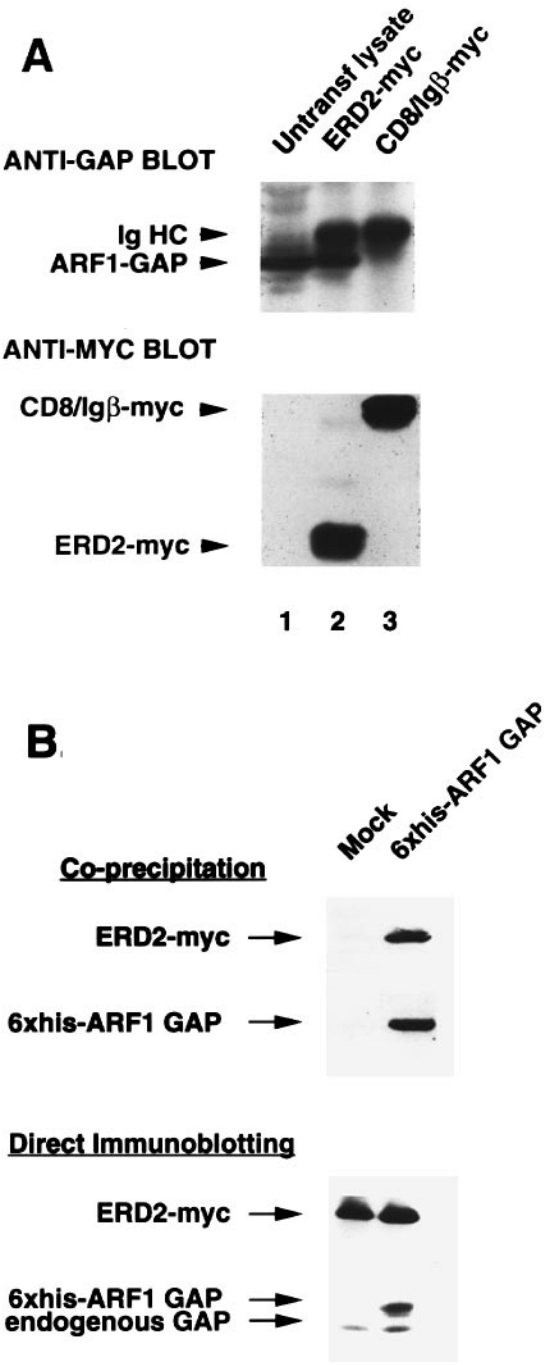

Fig. 4. ERD2 interacts with ARF1-GAP. (**A**) HeLa cells were transiently transfected with either ERD2-myc or a control myc-tagged construct (chimeric CD8), and then lysed and immunoprecipitated using an anti-myc antibody (9E10) followed by immunoblotting using either an anti-ARF1 GAP antiserum (upper panel, lanes 2 and 3) or an anti-myc antibody (lower panel, lanes 2 and 3). Direct immunoblotting of untransfected cell lysate with anti-ARF1 GAP antiserum reveals the relative migration of GAP (upper panel, lane 1). Ig HC, immunoglobulin heavy chain. (**B**) A HeLa cell line that stably expresses ERD2-myc was either mock or transiently transfected with 6×his-GAP, and then lysed and immunoprecipitated using an anti-6×his antibody followed by immunoblotting with either an anti-ARF1 GAP antiserum or an anti-myc antibody (upper panels). Direct immunoblotting using either of the two antibodies reveals relative levels of GAP or ERD2-myc in whole cell lysate (lower panels).

As a control that ERD2 oligomerization and its interaction with GAP are specific, we examined ERGIC53, an integral membrane protein that also self-oligomerizes and cycles between the ER and the Golgi complex (Lippincott-Schwartz *et al.*, 1990; Itin *et al.*, 1995). Overexpression of ERGIC53 did not result in the phenotype of ERD2 overexpression, i.e. β-COP remained in its Golgi-like distribution (Figure 7, upper panels), and Golgi complexes remained as distinct entities separate from the ER (Figure 7, lower panels). Accordingly, overexpressed ERGIC53 associated with neither GAP (Figure 6A, upper panel) nor ERD2 (Figure 6B, upper panel).

ARF1 GAP mediates the ability of ERD2 to induce a phenotype of ARF1 inactivation

The finding that ERD2 oligomerizes enabled us to ask whether there exists a mutant ERD2 that oligomerizes with wild-type ERD2, but does not allow these oligomers to associate with GAP. If such a mutant behaves in a dominant-negative fashion over the actions of wild-type ERD2, then this result suggests that GAP mediates the ability of overexpressed ERD2 to inactivate ARF1. Serial truncations from the C-terminus of ERD2 were performed, and two of these HA-tagged mutants were examined in more detail. One had the cytoplasmic tail of ERD2 deleted (7TM), while the other had an additional deletion of both the sixth and seventh transmembrane domains (5TM) (Figure 8A). Co-precipitation studies, performed by immunoprecipitating for HA-tagged constructs followed by immunoblotting for ERD2-myc, revealed that both mutants associated with wild-type ERD2 similarly to the way in which wild-type ERD2 self-associated (Figure 8B). However, 7TM complexes exhibited partial reduction in their interaction with GAP, while 5TM complexes had completely abrogated interaction with GAP (Figure 8C).

These mutants, when overexpressed alone, had reduced ability to induce a phenotype of ARF1 inactivation, as assessed by their ability to induce Golgi redistribution to the ER through the Tac-E19 assay (not shown). More significantly, when they were co-overexpressed with wild-type ERD2, the 7TM mutant caused a moderate reduction in Tac-E19 endo H resistance, while the 5TM mutant markedly reduced Tac-E19 endo H resistance (Figure 8D). As the degree to which ERD2 mutants blocked the effects of wild-type ERD2 correlated well with the degree to which they abrogated interaction with GAP, these findings suggested that ERD2 mutants exerted their dominant-negative effects on wild-type ERD2 by sequestering wild-type ERD2 into complexes that could not associate with GAP. Indeed, the co-overexpression of 5TM and wild-type ERD2 resulted in a reduced interaction between wild-type ERD2 and GAP, while the co-overexpression of a control construct had no such effect (Figure 8E). Thus, the behavior of the ERD2 mutants suggested that ERD2 oligomerization and its interaction with GAP were mechanistically linked and that this interaction mediated the ability of ERD2 to inactivate ARF1.

ERD2 regulates the recruitment of cytosolic GAP to membranes

As GAP can reside on membranes and in the cytosol (Cukierman *et al.*, 1995), the finding that the integral membrane protein ERD2 interacted with GAP suggested

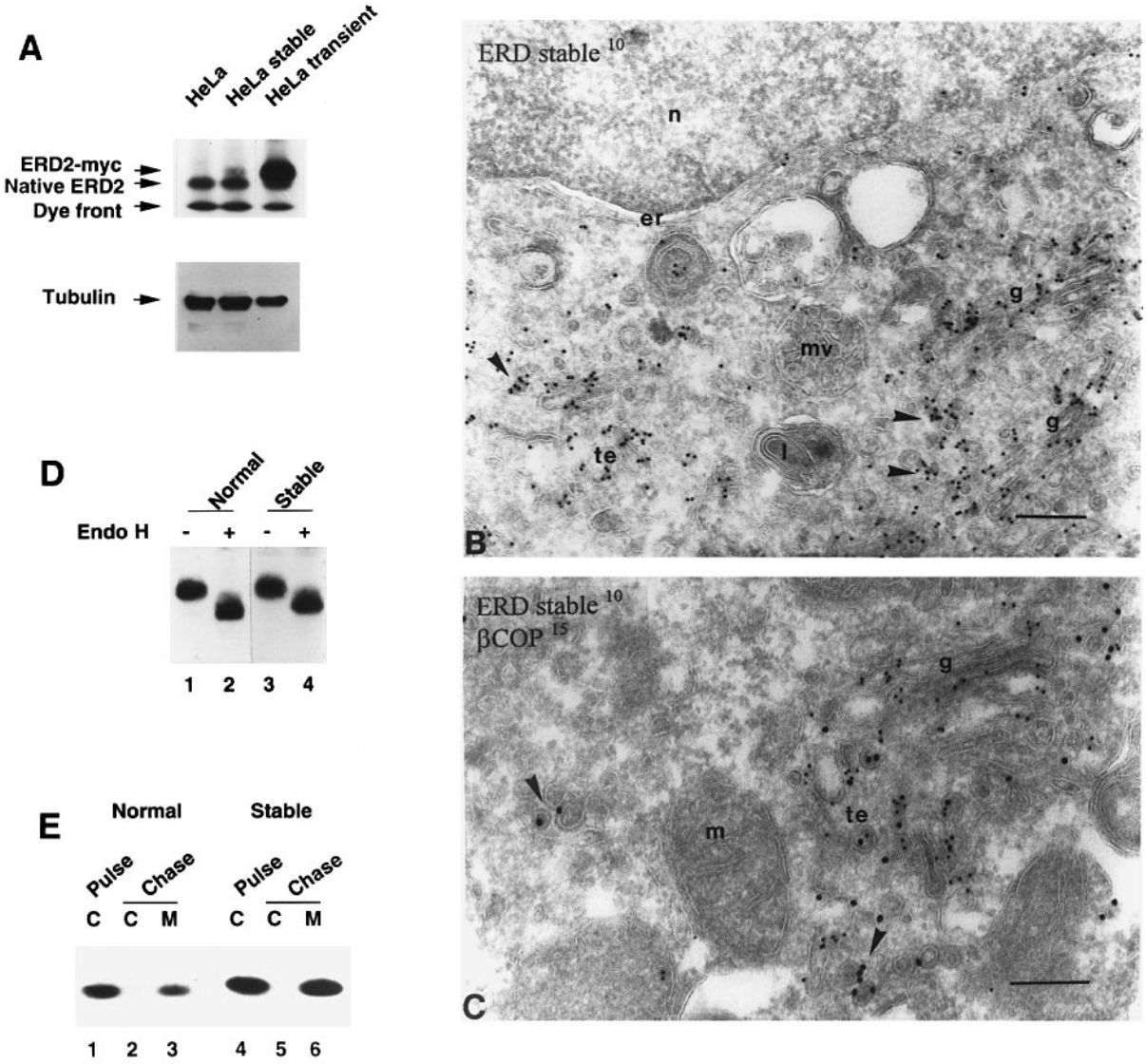

Fig. 5. A cell line that stably expresses ERD2-myc exhibits normal intracellular traffic. (**A**) The level of ERD2 expression differs in cell populations that are either transiently transfected or stably transfected with ERD2-myc. HeLa cells untransfected, stably transfected or transiently transfected with ERD2-myc were metabolically labeled with [^{35}S]methionine, lysed and immunoprecipitated with an antibody against native ERD2 (upper panel). Whole cell lysates were immunoblotted for tubulin to show similar cell equivalents evaluated (lower panel). (**B**) Stably expressed ERD2-myc in HeLa cells has an intracellular distribution similar to that of endogenous ERD2. By immunogold EM, labeling with anti-myc (10 nm gold) shows ERD2-myc in the Golgi stacks, coated vesicles (arrowheads) and transitional elements of the ER, a distribution similar to that described for endogenous ERD2. Bar, 200 nm; n, nucleus; er, ER; te, transitional ER; g, Golgi complex; mv, multivesicular body; l, lysosome. (**C**) Distribution of β-COP is normal in HeLa cells that stably express ERD2-myc. By immunogold EM, labeling with anti-β-COP (15 nm gold) reveals that β-COP is distributed normally on transitional elements of the ER, buds at the rims of the Golgi stacks and coated vesicles (arrowheads). ERD2-myc distribution is indicated by anti-myc (10 nm gold). Bar, 200 nm; e, ER; te, transitional ER; g, Golgi complex; m, mitochondrion. (**D**) The Golgi complex remains intact in ERD2-myc stable transfectants. Either normal HeLa cells (lanes 1 and 2) or those stably expressing ERD2-myc (lanes 3 and 4) were transiently transfected with Tac-E19, and then assessed for Golgi-specific glycosylation of Tac-E19. (**E**) Secretion is normal in ERD2-myc stable transfectants. Either normal HeLa cells (lanes 1–3) or those stably expressing ERD2-myc (lanes 4–6) were transfected with lysozyme, and assessed for lysozyme secretion; cells (C) and media (M).

that the distribution of GAP between membranes and cytosol might be regulated by this interaction. To test this hypothesis, we first examined whether ERD2 overexpression led to increased recruitment of GAP onto membranes. By subcellular fractionation, ERD2 overexpression led to an increase in the membrane-bound pool of GAP, with a corresponding decrease in its cytosolic pool (Figure 9). The increased GAP on membranes represented its recruitment from the cytosol and not its increased synthesis, because the total pool of GAP did not change upon ERD2 overexpression (Figure 9).

As transient transfection of ERD2-myc resulted in heterogeneous levels of ERD2 expressed from cell to cell, this variation enabled us to examine individual transfected cells by immunogold EM as a way of assessing how the level of ERD2 expression affected the recruitment of GAP to membranes. Compared with untransfected cells, cells that expressed moderate levels of ERD2-myc had increased GAP recruitment to membranes, and maintained distinct Golgi complexes (Figure 10A, upper panel). However, cells that greatly overexpressed ERD2 had even greater recruitment of GAP to membranes, but no longer exhibited

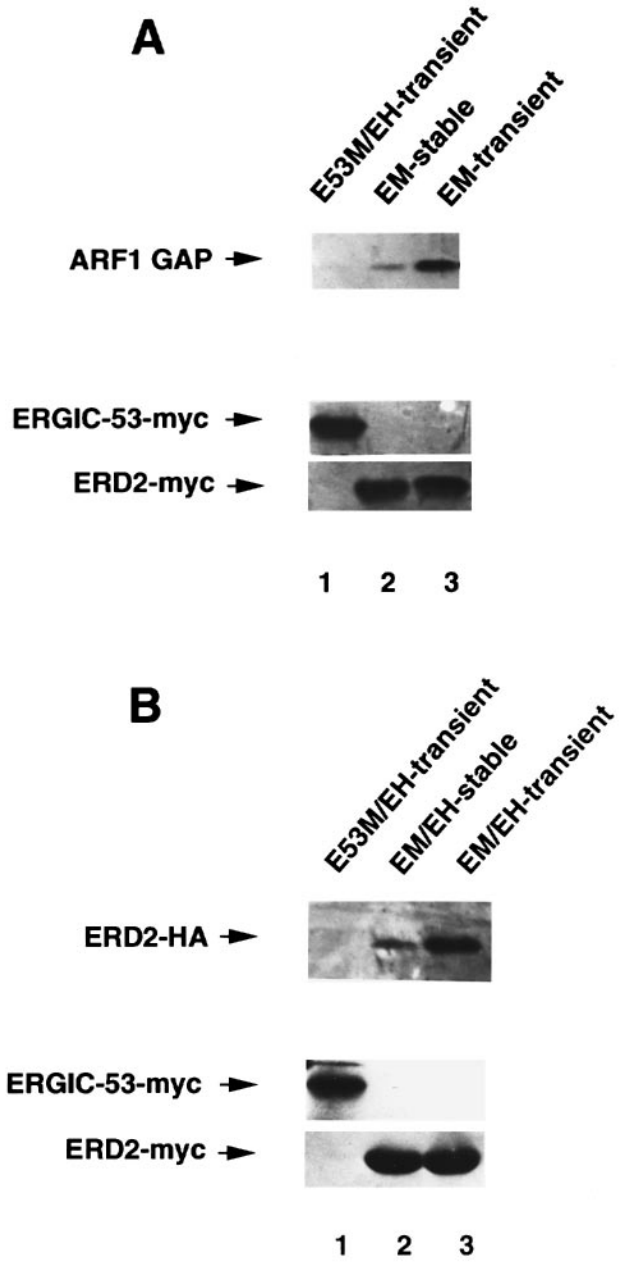

Fig. 6. ERD2 oligomerization and its interaction with GAP are enhanced upon ERD2 overexpression. (**A**) The interaction of ERD2 and GAP is specifically enhanced upon ERD2 overexpression. HeLa cells were transiently transfected with ERD2-myc (EM-transient), ERGIC53-myc and ERD2-HA (E53M/EH-transient) or stably transfected with ERD2-myc (EM-stable), and then lysed, immunoprecipitated with anti-myc antibody and immunoblotted with either anti-ARF1 GAP antiserum (upper panel) or anti-myc antibody (lower panels). (**B**) ERD2 oligomerization is enhanced upon ERD2 overexpression. HeLa cells were transiently transfected with ERD2-myc and ERD2-HA (EM/EH-transient), ERGIC53-myc and ERD2-HA (E53M/EH-transient), or stably transfected with ERD2-myc and ERD2-HA (EM/EH-stable), and then lysed, immunoprecipitated with anti-myc antibody and immunoblotted with either anti-HA antibody (upper panel) or anti-myc antibody (lower panels). The anti-myc antibody was covalently cross-linked to protein A–Sepharose beads to prevent the immunoglobulin heavy chain from obscuring ERGIC53-myc on immunoblots.

intact Golgi complexes (Figure 10B, lower panel). Quantitation of labeling for ERD2-myc and GAP revealed that GAP recruitment to membranes in cells that greatly

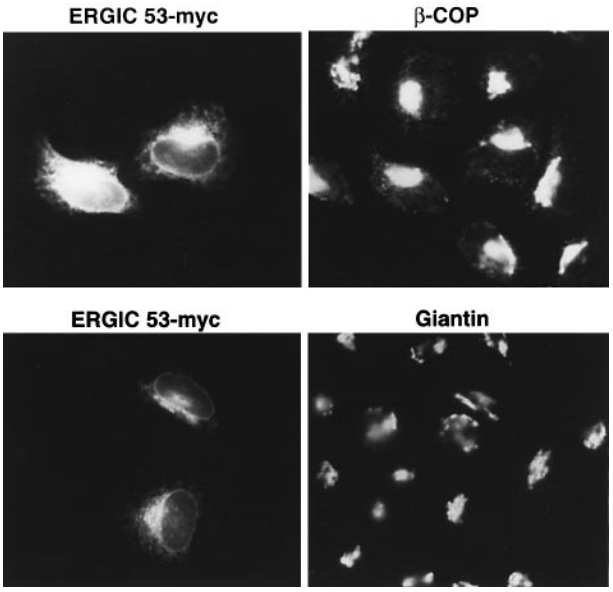

Fig. 7. Overexpression of ERGIC53 does not result in the phenotype of ARF1 inactivation. COS cells were transiently transfected with ERGIC53-myc and evaluated by immunofluorescence microscopy with double labeling using either mouse anti-myc antibody and rabbit anti-β-COP (upper panels) or mouse anti-myc antibody and rabbit anti-giantin antibody (lower panels).

overexpressed ERD2 was enhanced 8-fold over GAP recruitment in cells with moderately expressed ERD2 (Table II). Thus, these results suggested that the level of ERD2 determined GAP recruitment to membranes and the ability of ERD2 to induce a phenotype of ARF1 inactivation.

Discussion

ERD2 regulates ARF1 through ARF1 GAP

This study suggests a mechanism by which ERD2 regulates transport in the early secretory system. A crucial insight into this mechanism is the observation that the overexpression of either ERD2 or GAP results in a common phenotype that appears specific for inactivated ARF1. This finding is seen in many different cell types that we have examined, including COS, HeLa, HEK 293 and RD4 cell lines (not shown), and suggests the possibility that the actions of ERD2 and GAP are linked. Indeed, we detect an interaction between ERD2 and GAP that is enhanced upon ERD2 overexpression. However, as this interaction is detected by a co-precipitation approach, it remains to be determined whether this interaction is direct. Nevertheless, this interaction mediates the ability of overexpressed ERD2 to inactivate ARF1, because a mutant ERD2 blocks the ability of wild-type ERD2 to induce a phenotype of ARF1 inactivation by abrogating the interaction of wild-type ERD2 with GAP. To understand how GAP mediates the effects of ERD2, we examine transiently transfected cells that express varying levels of ERD2. With increasing ERD2, more cytosolic GAP is recruited onto membranes. At a critical level of enhanced GAP recruitment, a phenotype of ARF1 inactivation is observed. Thus, these findings suggest that ERD2 regulates ARF1-mediated transport by recruiting more cytosolic ARF1 GAP to membranes.

ERD2 regulates the recruitment of GAP to membranes

The translocation of cytosolic GAP to membranes probably enhances GAP function, as its substrate (ARF1-GTP) is membrane-restricted (Donaldson *et al.*, 1992; Palmer *et al.*, 1993), and previous studies suggest that the functional pool of GAP resides on membranes (Helms *et al.*, 1993; Ostermann *et al.*, 1993). However, we cannot assess directly whether ERD2 overexpression inactivates ARF1, for two reasons. First, GTP binding to ARF1 is unstable (Weiss *et al.*, 1989; Franco *et al.*, 1995), and thus, isolating ARF1 to directly measure changes in its ratio of GTP-bound to GDP-bound forms, as a reflection of changes in its activation status *in vivo*, would be unreliable. Second, GAP activity toward ARF1 is highly active in membrane fractions (Helms *et al.*, 1993; Ostermann *et al.*, 1993), and thus, GTPγS is required to lock ARF1 in its GTP-bound state to detect ARF1 on membranes (Donaldson *et al.*, 1992; Palmer *et al.*, 1993). However, as we can only examine ARF1 using GTP that is present in the cytosol, ARF1 invariably will be found to be mostly cytosolic upon subcellular fractionation. Hence, *in vitro* approaches cannot reliably measure the activation status of ARF1 *in vivo*.

To circumvent this problem, we use an alternate approach, as demonstrated by studies on other families of small GTPases (Lowy and Willumsen, 1993; Machesky and Hall, 1996). ERD2 overexpression results in a phenotype similar to that of ARF1 inactivation, which is characterized by the release of COPI from Golgi membranes to the cytosol, Golgi redistribution to the ER and a block of transport out of this fused membrane compartment (Hsu *et al.*, 1992). Other perturbations share or resemble superficially some aspect of this phenotype (Donaldson *et al.*, 1991; Sugai *et al.*, 1992; Takizawa *et al.*, 1993; Warren, 1993; Nilsson *et al.*, 1994; Wilson *et al.*, 1994). However, only perturbations that affect the regulators of ARF1 induce all the manifestations described for this phenotype. Furthermore, the overexpression of ARF1-T31N, GAP or ERD2 all induce a similar phenotype that is reversed similarly by supplying more activated ARF1 to cells through ARF1 overexpression. Thus, we use this phenotype to demonstrate that recruiting more cytosolic GAP to membranes upon ERD2 overexpression results in enhanced ARF1 inactivation, suggesting that GAP recruitment from the cytosol to membranes provides a mechanism for regulating its function.

A proposed signaling process regulating transport

Our description of how ERD2 affects transport suggests a regulatory process that bears a remarkable resemblance to a widely used signaling process, as exemplified by how growth factors initiate intracellular signaling through their receptors (Heldin, 1995). In both cases, it appears that membrane-bound receptors oligomerize and recruit cytosolic effectors to membranes, and this recruitment is a mechanism for regulating the function of the effectors. Thus, despite intracellular transport and growth factor

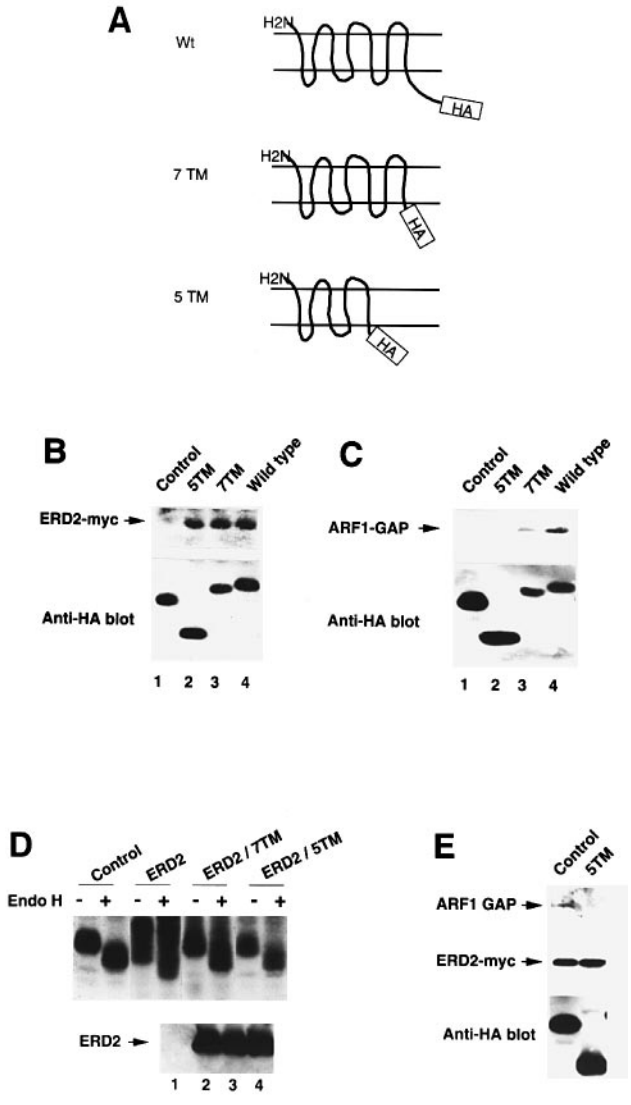

Fig. 8. GAP mediates the ability of ERD2 to inactivate ARF1. (**A**) A schematic representation of the ERD2 mutants generated by PCR mutagenesis. Wt, wild-type; 7TM, mutant with cytoplasmic tail deleted; 5TM, mutant with the last two transmembrane domains and cytoplasmic tail deleted; HA, HA epitope tag. (**B**) ERD2 mutants oligomerize with wild-type ERD2 similarly to the way in which wild-type ERD2 oligomerizes with itself. HeLa cells were transiently transfected with ERD2-myc in combination with a control construct (ARF6-HA) (lane 1), 5TM-HA (lane 2), 7TM-HA (lane 3) or ERD2-HA (lane 4), lysed and immunoprecipitated with an anti-HA antibody followed by immunoblotting with an anti-myc antibody (upper panel) or an anti-HA antibody (lower panel). (**C**) ERD2 mutants have reduced interaction with GAP. HeLa cells were transiently transfected with a control construct (ARF6-HA) (lane 1), 5TM-HA (lane 2), 7TM-HA (lane 3) or ERD2-HA (lane 4), lysed, immunoprecipitated with an anti-HA antibody followed by immunoblotting with an anti-ARF1 GAP antiserum (upper panel) or an anti-HA antibody (lower panel). (**D**) Co-overexpression of mutant ERD2 blocks Golgi redistribution to the ER induced by the overexpression of wild-type ERD2. HeLa cells were transiently transfected with Tac-E19 (upper panel) in combination with control construct, wild-type ERD2-myc and control construct, ERD2-myc and 7TM-HA, or ERD2-myc and 5TM-HA, and then assessed for Golgi-specific glycosylation of Tac-E19. Direct immunoblotting of whole cell lysates with anti-myc antibody revealed similar degrees of wild-type ERD2-myc overexpression (lower panel). (**E**) Co-overexpression of 5TM reduces the interaction of overexpressed ERD2 with GAP. HeLa cells were transiently transfected with wild-type ERD2-myc and either 5TM-HA or control construct (ARF6-HA), and then lysed, followed by immunoprecipitation for ERD2-myc using an anti-myc antibody and immunoblotting with either an anti-ARF1 GAP antiserum (upper panel) or an anti-myc antibody (middle panel). Direct immunoblotting of whole cell lysates using an anti-HA antibody reveals equal levels of 5TM and control expressed (lower panel).

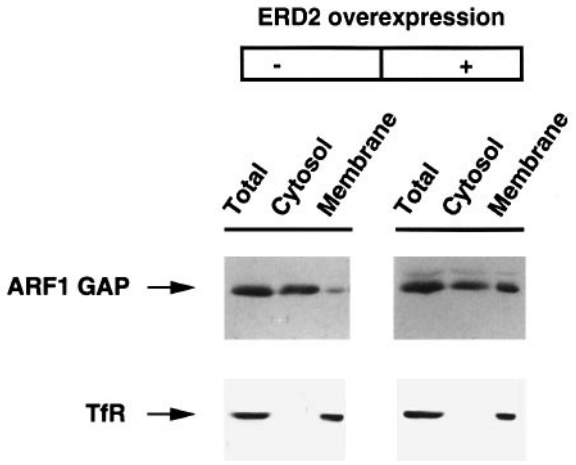

Fig. 9. ERD2 overexpression recruits cytosolic ARF1 GAP to membranes. HeLa cells were transiently transfected with ERD2-myc and subjected to subcellular fractionation. Cellular fractions (total, cytosolic or membrane) were immunoblotted for ARF1 GAP (upper panel), and also for transferrin receptor (TfR) (lower panel) to show quantitative recovery of total membranes.

receptor signaling being two very different cellular processes, they apparently share a fundamentally similar mechanism of regulation. This analogy leads us to propose that overexpressed ERD2 represents a constitutively activated receptor that self-oligomerizes to interact with GAP, and thereby shifts more cytosolic GAP to its functional pool on membranes. This proposal suggests that the KDEL proteins may normally instigate the regulation of GAP

Table II. Overexpression of ERD2 results in increased ARF1 GAP on membranes

Golgi profiles	ERD2-myc/unit membrane	No. of ARF1-GAP/ unit membrane
Intact	<15 (moderately overexpressed)	1.8 ± 0.8
Not intact	>40 (greatly overexpressed)	14.7 ± 2.1

Transfected cells were determined for intact Golgi profiles, and then assessed for ERD2-myc expression by anti-myc labeling (5 nm gold) along the unit length of nuclear ER per cell. At a calibrated magnification, labeling for ARF1 GAP (10 nm gold) along a unit length of nuclear ER per cell was counted. The statistical difference in the level of ARF1 GAP detected was $P < 1 \times 10^{-9}$.

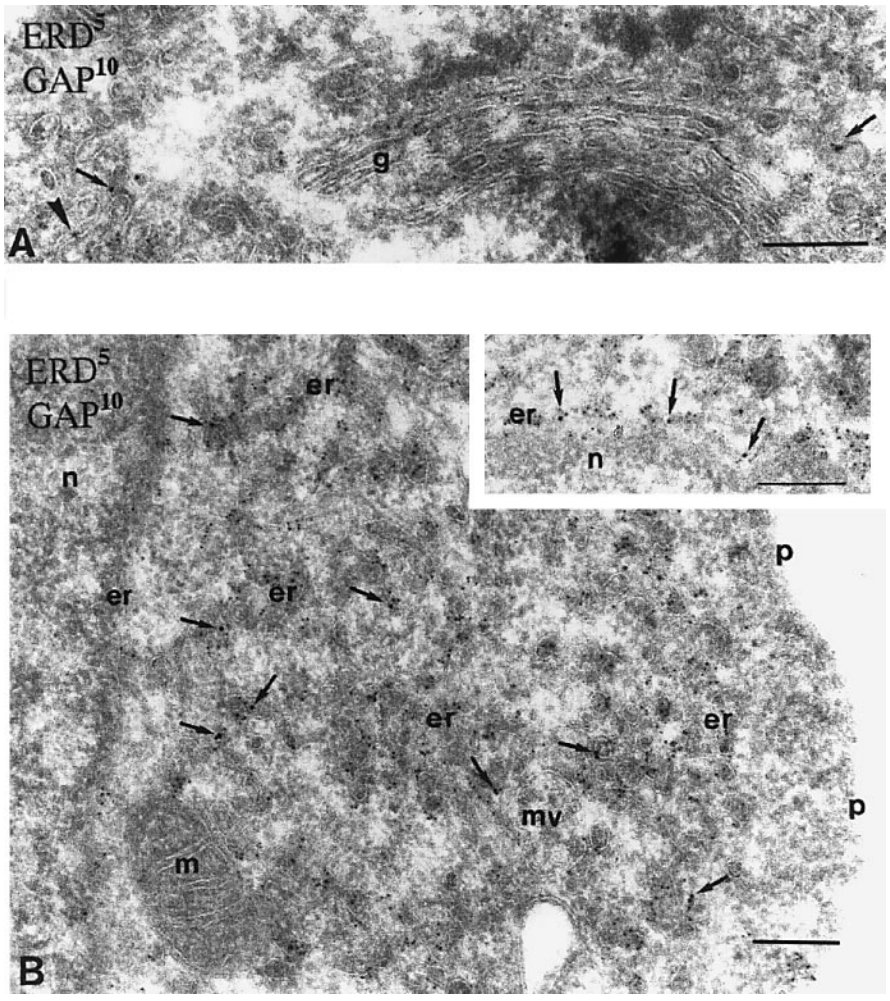

Fig. 10. Level of ERD2 expression determines the degree of GAP recruitment to membranes and the ability of ERD2 to induce the phenotype of ARF1 inactivation. HeLa cells were transiently transfected with ERD2-myc and then evaluated by immunogold EM with double labeling using anti-myc antibody (5 nm gold) and anti-ARF1 GAP antiserum (10 nm gold); bar, 200 nm; n, nucleus; er, ER; mv, multivesicular body; p, plasma membrane; m, mitochondrion. (**A**) Low expressors of ERD2 have moderately increased GAP on membranes (arrows) and intact Golgi complexes. (**B**) High expressors of ERD2-myc have greatly increased GAP on membranes (arrows) including the nuclear envelope (see inset), and no intact Golgi complexes.

from the luminal side of transport pathways. Consistent with this possibility, ERD2 oligomerization and its interaction with GAP that are seen under physiologic circumstances, when ERD2 is not overexpressed, may reflect ongoing modulation of ERD2 by endogenous KDEL proteins. The elucidation of such a possibility will provide a greater understanding of the different mechanisms by which intracellular traffic may be regulated.

Materials and methods

Cells and antibodies

COS-7 and HeLa cells were grown in complete medium that consisted of Dulbecco's modified essential medium (DMEM; Life Technologies, Inc., Gaithersburg, MD) with 10% fetal calf serum (FCS), 2 mM glutamine, 40 μg/ml gentamicin at 37°C in a 5% CO_2 incubator.

The following antibodies were used: mouse monoclonal antibody (mAb) 9E10 against the myc epitope (ATCC, Rockville, MD), mouse mAb 15E6 against the hemagglutinin (HA) epitope, mouse mAb against the 6×his epitope (Clontech, Palo Alto, CA), mouse mAb F10.6.6 against lysozyme (provided by R.Poljak, Paris, France), mouse mAb 7G7 against Tac antigen (provided by D.Nelson, Bethesda, MD), mouse mAb M3A5 against β-COP (provided by T.Kreis, Geneva, Switzerland), mouse mAb against ERD2 (provided by W.Hong and B.L.Tang, Singapore), mouse mAb against transferrin receptor (provided by I.Trowbridge), rabbit polyclonal anti-β-COP antiserum (provided by J.Lippincott-Schwartz, Bethesda, MD), rabbit polyclonal anti-ARF1 GAP antiserum (Cukierman *et al.*, 1995) and rabbit polyclonal anti-giantin antiserum (provided by M.Renz, Karlsruhe, Germany).

Fluorescein-conjugated and indocarbocyanine-conjugated donkey anti-mouse and anti-rabbit IgGs were obtained from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Rhodamine-conjugated *Lens culinaris* lectin was obtained from E.Y.Laboratories (San Mateo, CA).

Plasmids and stable transfectants

The following cDNAs were used. HA- and myc-tagged human ERD2, lysozyme and chimeric Tac-E19 have been previously described (Hsu *et al.*, 1992). HA-tagged ARF6, ARF1 and ARF1-T31N have also been described (Peters *et al.*, 1995). 6×his-tagged ARF1 GAP was generated by subcloning cDNA encoding ARF1 GAP into pcDNA3.1/His (Invitrogen, Carlsbad, CA) that confers the 6×his epitope onto the N-terminal end of ARF1 GAP. Myc-tagged CD8/Igβ (consisting of the extracellular and transmembrane domains of CD8, and the cytoplasmic tail of Igβ followed by the myc tag) was kindly provided by H.Band (Boston, MA). Myc-tagged ERGIC53 was kindly provided by H.-P.Hauri (Basel, Switzerland).

Truncation of ERD2 from the C-terminus to generate ERD2 mutants was performed by PCR. The 5′ primer used consists of the sense sequence for an *Eco*RI site followed by bases 1–21 of the ERD2-coding sequence. The 3′ primer for the 5TM mutant consists of the antisense sequence for bases 406–426 of the ERD2-coding sequence followed by codons for the HA tag and an *Xba*I site. The 3′ primer for the 7TM mutant consists of the antisense sequence for bases 189–209 of the ERD2-coding sequence followed by codons for the HA tag and an *Xba*I site. The amplified sequences were subcloned into the mammalian expression vector pCDLSRα (Takebe *et al.*, 1988) and verified by DNA sequencing using the Sequenase kit (US Biochemical Corp, Cleveland, OH).

Transfection with the calcium phosphate method was performed as previously described (Hsu *et al.*, 1992). To generate stable cell lines, myc-tagged ERD2, or both myc-tagged and HA-tagged ERD2 constructs were co-transfected with a construct containing the neomycin resistance gene (pRSV.5NEO) (Frank *et al.*, 1990) into HeLa cells by the calcium phosphate method. Cells were selected in complete medium containing 1 mg/ml of G418 (Life Technologies, Inc., Gaithersburg, MD). Stable transfectants were screened by immunofluorescence microscopy.

Immunofluorescence microscopy

Cells on coverslips were fixed in 2% formaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature, and then processed as previously described (Hsu *et al.*, 1992). Coverslips were mounted with fluoromount G (Electron Microscopy Science, Washington, PA). Microscopy (Nikon Cooperation, Tokyo, Japan) was performed with 40× or 60× oil planapo lens.

Electron microscopy

The procedures used were described previously (Peters *et al.*, 1991). Briefly, samples were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h at room temperature. Cells were transferred in 0.2% paraformaldehyde, scraped, collected and embedded in 10% gelatin at 4°C. Samples were processed for cryoelectron microscopy using a Leica Ultracryomicrotome and Drukker diamond knife (Drukker International, Cuijk, The Netherlands). Sections of 50 nm were cut at –120°C and collected with a mixture of sucrose and methyl cellulose (Liou *et al.*, 1996). Cryosections were incubated at room temperature with antibodies for 30 min, washed and incubated for 20 min with protein A–gold. As a specificity control, only transfected cells showed labeling using anti-HA, anti-myc or anti-ARF1 GAP antibody.

Immunoprecipitation and immunoblotting

Transfected cells were removed from dishes, pelleted by centrifugation and lysed for 30 min at 4°C in a buffer containing 1% Triton X-100, 50 mM Tris–HCl pH 7.4, 300 mM NaCl, 10 μg/ml aprotinin and 10 μg/ml leupeptin. Lysates were pre-cleared with protein A–Sepharose beads, and incubated with antibody-coupled protein A–Sepharose for 1 h at 4°C. Immunoprecipitates were washed, boiled in sample buffer and separated by SDS–PAGE under reducing conditions. For immunoblotting, gels were transferred onto polyvinylidene fluoride membranes (Immobilon-P, Millipore Corp., Bedford, MA), blocked, incubated with primary antibodies followed by peroxidase-conjugated donkey anti-mouse or anti-rabbit IgG antibody, and developed by chemiluminescence (Renaissance, DuPont NEM Research Products, Boston, MA).

Golgi-specific glycosylation of Tac-E19

Cells were transiently transfected with the Tac-E19 construct alone or with other constructs as described in the figure legends. Twenty four hours later, cells were pre-incubated in labeling medium (DMEM without methionine supplemented with 2% FCS, 2 mM glutamine and 40 μg/ml gentamicin) for 20 min at 37°C, labeled with [^{35}S]methionine (DuPont NEM Research Products, Boston, MA) at 100 μCi/ml for 2 h, washed and then chased in complete medium for 16 h. Cells were removed from dishes, lysed and pre-cleared with protein A–Sepharose beads for 30 min. The pre-cleared supernatant was then incubated at 4°C for 1 h with anti-Tac antibody previously coupled to protein A–Sepharose. Beads were either mock treated or treated with endo H (New England Biolabs, Beverly, MA) for 2 h at 37°C. Samples were then analyzed by SDS–PAGE.

Lysozyme secretion assay

Cells were transiently transfected with lysozyme vector alone and/or with other constructs. Forty hours later, cells were pre-incubated in labeling medium for 20 min at 37°C, labeled with [^{35}S]methionine at 100 μCi/ml for 30 min, washed and then chased in complete medium for 4 h. After collecting the media, cells were removed from dishes, lysed and pre-cleared with protein A–Sepharose. Media and cell lysates were incubated at 4°C for 1 h with anti-lysozyme antibody, F10.6.6, previously coupled to protein A–Sepharose. After washing, samples were analyzed by SDS–PAGE.

Subcellular fractionation

HeLa cells were transiently transfected and, 40 h later, cells were scraped and washed with PBS twice and with homogenization buffer (10 mM triethanolamine pH 7.4, 250 mM sucrose, 1 mM EDTA) once. The cells were homogenized by four passes through a ball-bearing homogenizer. Nuclei and cell debris were removed by centrifugation at 800 *g* for 10 min. The post-nuclear supernatant was centrifuged for 1 h at 200 000 *g* to obtain membrane and cytosolic fractions.

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