Erf4p and Erf2p Form an Endoplasmic Reticulum-associated Complex Involved in the Plasma Membrane Localization of Yeast Ras Proteins

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Ras oncogene proteins are plasma membrane-associated signal transducers that are found in all eukaryotes. Posttranslational addition of lipid to a carboxyl-terminal Caax box (where “C” represents a cysteine, “a” is generally an aliphatic residue, and X can be any amino acid) is required to target Ras proteins to the cytosolic surface of the plasma membrane. The pathway by which Ras translocates from the endoplasmic reticulum to the plasma membrane is currently not clear. We have performed a genetic screen to identify components of the Ras plasma membrane localization pathway. Mutations in two genes, ERF2 and ERF4/SHR5, have been shown to affect the palmitoylation and subcellular localization of Ras proteins. In this report, we show that Erf4p is localized on the endoplasmic reticulum as a peripheral membrane protein in a complex with Erf2p, an integral membrane protein that was identified from the same genetic screen. Erf2p has been shown to be required for the plasma membrane localization of GFP-Ras2p via a pathway distinct from the classical secretory pathway (X. Dong and R. J. Deschens, manuscript in preparation). We show here that Erf4p, like Erf2p, is involved in the plasma membrane localization of Ras2p. Erf2p and Erf4p represent components of a previously uncharacterized subcellular transport pathway involved in the plasma membrane targeting of Ras proteins.

Ras proteins are plasma membrane-bound small GTPases that regulate signal transduction pathways by cycling between GTP- and GDP-bound forms (1, 2). Ras proteins are initially synthesized as cytosolic precursors, but then undergo modifications at a carboxyl-terminal motif called the Caax box (where “C” represents a cysteine, “a” is generally an aliphatic residue, and “X” can be any amino acid) (3). These modifications include farnesylation of the Caax box cysteine, proteolysis of the -aaX, and carboxyl methylation (4–8). The last two steps occur on the cytosolic surface of the ER.1 Most Ras proteins, including yeast Ras1p and Ras2p and mammalian H-Ras and N-Ras, are further modified by palmitoylation on one or two additional cysteine residues often found adjacent to the Caax box. Not all prenylated Ras proteins undergo palmitoylation. Mammalian K-Ras4B, for example, lacks a palmitoylation site but contains multiple basic residues near the C terminus that are required for plasma membrane targeting (4, 9–11). These observations have led to a two-signal hypothesis for trafficking in which Caax box processing plus at least one additional signal is required for plasma membrane localization of Ras (12).

The mechanism by which Ras and other prenylated proteins are transported from the cytoplasmic surface of the ER to the plasma membrane is not clear. The classical secretory pathway, which has been explored extensively by genetic studies in S. cerevisiae and biochemical fractionation of mammalian cell lines, is an obvious candidate (13–15). Many proteins are transported via the classical secretory pathway by a process of vesicle budding and fusion (16, 17). Lipid-anchored Ras proteins could be transported to the plasma membrane by hitchhiking on vesicles as they mature through the classical secretory pathway. However, not all protein trafficking from the ER to the plasma membrane depends on the classical secretory pathway. Examples of proteins and peptides that are either secreted or plasma membrane-localized independent of the classical secretory pathway include yeast a-factor (18, 19), Nee3/Nee103 (20), and, more recently, mammalian K-Ras (11). Brefeldin A blocks the transport of GFP fused to the C-terminal sequences of H-Ras, but a similar construct with the C terminus of K-Ras is not affected by brefeldin A (12, 21). These observations have created an interest in defining the nonclassical pathway by which Ras and other prenylated proteins reach the plasma membrane.

We have previously described two yeast proteins, Erf2p and Erf4p, involved in the palmitoylation and subcellular localization of yeast Ras protein (22). In this report, we show that Erf4p, like Erf2p, is localized on the ER and interacts directly with Erf2p. Previously, we have shown that the plasma membrane localization of GFP-Ras2p does not require the classical secretory pathway but involves an alternative pathway that requires Erf2p.2 The Erf2p-Erf4p complex is a palmitoyl S-acyltransferase for the yeast Ras proteins (23). Together these results demonstrate that palmitoylation is required for the ER to plasma membrane translocation of Ras proteins in yeast. The nature of this palmitoylation-dependent Ras subcellular localization pathway will be discussed.

EXPERIMENTAL PROCEDURES

Strains and Plasmids—Strains used in this study are listed in Table I. Deletion of ERF4 was performed by homologous recombination of a kanamycin-resistant gene flanked by 50 nucleotides identical to sequences upstream and downstream of the ERF4 open reading frame, respectively. The knockout cassette was amplified by PCR from pUG6

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2 X. Dong and R. J. Deschenes, manuscript in preparation.

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Table I

| Strains used in this study | Strain | Genotype | Reference |
|---------------------------|--------|----------|-----------|

LRB933 MATa his3 leu2 ura3–52 sec14
LRB937 MATa his3 leu2 ura3–52 sec23
LRB938 MATa his3 leu2 ura3–52
PJ69-4A MATa trp1–901 leu2–3,112 ura3–52 his3–500 gal4Δ gal80Δ GAL2–ADE2 LYS2: GAL1–HIS3 met2: GAL7–lacZ

RJY266 MATα leu2–3,112/trp1–901 ura3–52/his3Δ111 /his3Δ111 trp1–289/trp1–289

RJY67 MATa trp1–901 leu2–3,112 ura3–52/aadA8 lys2–1

RJY107 MATa trp1–901 leu2–3,112 ura3–52/aadA8 lys2–1

RJY104 MATa trp1–901 leu2–3,112 ura3–52/aadA8 lys2–1

RJY1054 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY104 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY1090 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY1096 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY1277 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY1294 MATa his3 leu2 ura3–52/hrf4–1: KanR

RJY1543 MATa his3 leu2 ura3–52/hrf4–1: KanR

* Extention (ext) is used as an abbreviation for the amino acid sequence IIKLIKRK.
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goat anti-rabbit horseradish peroxidase were commercially available from Amersham and used as a 1:1,000 dilution in 5% milk in buffer A. The Western blots were then visualized with a Pierce SuperSignal kit.

**Sucrose Gradient Fractionation**—Sucrose gradient fractionation was performed essentially as described (34). Briefly, NaCl (10 mM) and KF (10 mM) were added to overnight cell cultures, and the cells were harvested, washed in buffer containing 10 mM NaCl, 10 mM KF, and 5 mM Tris-HCl, pH 7.6; and resuspended in either STE10 (10% sucrose; 10 mM Tris-HCl, pH 7.6; 2 mM Mg2+). Cells were broken with glass beads, and the postnuclear supernatant (700 μl) was collected as described above, loaded on a 20–60% linear sucrose gradient containing 10 mM EDTA, and subjected to centrifugation in a SW41 rotor (Beckman) at 28,400 rpm for 18 h. Fractions (600 μl) were taken from the top, and the proteins were resolved by SDS-polyacrylamide gel, followed by Western blot. The postnuclear supernatant prepared in STM10 was processed likewise, but the linear gradient contains 2 mM Mg2+, without EDTA. Rabbit anti-Sec61p IgG was generously provided by Dr. Scott Moye-Rowley and used at a 1:5,000 dilution as described (35). Goat anti-rabbit IgG horseradish peroxidase conjugate was purchased from Sigma and diluted 1:3,000 before use.

**Immunofluorescence and GFP Subcellular Localization Experiments**—An immunofluorescence experiment was performed as described previously (22). Briefly, 10 ml of early log phase (Ano~0.3–0.5) cell culture was harvested by centrifugation at 9000 × g and fixed in 0.1 M potassium phosphate buffer, pH 6.5, containing 4.4% formaldehyde. After fixation, the cells were washed, and spheroplasts were prepared by treating with zymolysate (15 μg/ml) in 0.1 M potassium phosphate buffer, pH 6.5, containing 1.2 mM sorbitol. Spheroplasts were spotted on glass slides and incubated with mouse anti-HA IgG (1:1,000) and rabbit anti-Sec61p IgG (1:5,000) and detected by goat anti-mouse IgG-Alphal horseradish conjugate (1:240, Molecular Probes) and goat anti-rabbit IgG-rhodamine conjugate (1:240, Jackson ImmunoResearch Laboratories), respectively.

For experiments involving GFP fusion proteins, the following strains were used: LR9983, RYJ1543, LR9837, RYJ1544, LR9893, and RYJ1545 (Table 1). The cells were transformed with Yepl565-GFP-ERF4. Transformants were inoculated into synthetic complete lacking leucine (SC-Leu), supplemented with 2% ethanol and 2% glycerol as carbon sources. The cells were grown at 24°C until reaching early log phase (Ano~0.2–0.4). Galactose was added to the culture to a final concentration of 4% to induce GFP-Ras expression for 4 h at either 24 or 37°C. Samples were analyzed by confocal microscopy (60× objective, MRC-1024, Bio-Rad).

**Genetic Studies and Two-hybrid Assay**—The genetic screen to identify erf mutants was described previously (22). For high copy suppression test, the mutant erf2 strains were transformed with Yeplac112-ERF4 and grown on a synthetic complete medium lacking tryptophan and uracil (SC-Leu-Ura). Transformants were transferred to SC-Leu-Ura plates supplemented with 1 mg/ml 5-fluoroorotic acid. Similarly, mutant erf4 strains were transformed with Yeplac112-ERF4 and processed as described above.

For two-hybrid assays, bait and prey plasmids were introduced into PJ69-4A strain (38). Erf4p was expressed from pAS1-CYH2 (bait), fused with the Gal4 DNA-binding domain and an HA epitope tag, or expressed from pGAD (prey) as a fusion protein with a Gal4 transcription activation domain. Erf2p was expressed from pGBD (bait) as a fusion protein with the Gal4 DNA-binding domain or expressed from pGAD (prey). The transformants were grown on synthetic complete plates lacking tryptophan and leucine (SC-Leu-Trp) and then replicated onto 3-aminotriazole plates (SC-Leu-Trp-His, supplemented with 3 mM 3-aminotriazole). The transformants were then incubated on plates lacking tryptophan and leucine (SC-Leu-Trp) and then replicated.

**GST Affinity Chromatography and FLAG Immunoprecipitation**—Yeast cells of YPH499 with pESC-TRP-ERF2 and either pEG(KT) or pEG(KT)-ERF4 were collected after galactose induction and lysed in Y-PER solution (Pierce) according to the manufacturer’s protocol. For GST affinity chromatography, cell extract was incubated with GSH-agarose beads for 30 min at room temperature. The beads were collected by centrifugation at 1000 × g and washed three times with 50 mM Tris-HCl, pH 7.4 (10 times the volume of GSH-agarose beads). The GST fusion proteins were eluted at room temperature for 1 h in 50 mM Tris-HCl, pH 7.4, with 20 mM glutathione and 0.02% Triton. For FLAG immunoprecipitation, protein extract was prepared in immunoprecipitation buffer (50 mM Tris-Cl, pH 7.4, 10 mM EDTA, 100 mM NaCl, 0.1% Triton X-100) and incubated with M2 anti-FLAG IgG-agarose beads (Sigma) at room temperature for 40 min. The beads were washed with immunoprecipitation buffer three times. GST fusion proteins were probed by rabbit anti-GST antibody (Molecular Probes) and peroxidase-conjugated goat anti-rabbit secondary antibody (Sigma) on the Western blot. Mouse anti-FLAG antibody (M5, Sigma) was used to detect FLAG-tagged Erf2p.

**RESULTS**

The Viability of Yeast Strains Expressing a Palmitoylation-dependent ras Allele Requires ERF4—Previously, we described the isolation of a set of palmitoylation-dependent yeast Ras proteins and a genetic screen for mutants that were inviable when the palmitoylation-dependent RAS2 allele is the only RAS gene expressed (22, 36). The mutations fell into two complementation groups. One gene, Erf2, encodes a Asp-His-Cys (DHHC)-zinc finger protein that is associated with the ER membrane and affects the palmitoylation and plasma membrane targeting of Ras2p (22). The other gene, Erf4, was previously identified as SHR5 (suppressor of hyperactive Ras), and null mutations in Erf4 also reduce Ras2p palmitoylation (22, 37). Because the acronym SHR has been used for genes involved in amino acid metabolism prior to the naming of SHR5 (38), we will refer the gene as Erf4.

Erf4p is predicted to encode a 26.5-kDa protein. Analysis of the Erf4p sequence reveals that the C terminus is rich in leucine residues, with one region predicted to adopt a leucine zipper motif (residues 197–218), and a second region forms a hydrophobic domain (residues 167–187) with a Kyte-Doolittle score of 1.44, lower than typically observed for a transmembrane domain (Fig. 1A). In addition to the putative leucine zipper and hydrophobic domains, a short stretch rich in aromatic amino acids is found close to the amino terminus (residues 20–31). Four mutations were identified in the original genetic screen. Alleles erf4–1 and erf4–2 are missense mutations S128P and V148K, respectively. One mutation, L204P (erf4–3) involves a leucine residue of the putative leucine zipper. A nonsense mutation at Trp180 (erf4–4) was also recovered from the screen (Fig. 1A). To date, it has not been possible to identify putative metazoan homologs of Erf4p by sequence homology searches.

To examine the effect of Erf4p on Ras function, the Erf4p gene was deleted in the same strain background on which the original genetic screen was performed. Similar to the mutations isolated from the screen, deletion of Erf4p caused a severe growth defect when combined with the palmitoylation-dependent RAS2 allele (Fig. 1B). The deletions as well as mutations isolated from the genetic screen are fully complemented by a low copy yeast plasmid expressing wild type Erf4p (Fig. 1C). Erf4p Is a Membrane Protein Associated with the Endoplasmic Reticulum—The existence of a hydrophobic region in Erf4p prompted us to examine whether Erf4p, like Erf2p, is membrane-associated. Differential fractionation experiments were performed using a 6× HA-tagged Erf4p (Erf4(6HA)p) expressed from a CEN plasmid under the control of the endogenous Erf4 promoter. Expression of Erf4(6HA)p fully complements an Erf4 null mutation (data not shown). The postnuclear supernatant was fractionated by centrifugation at 100,000 × g to prepare membrane and soluble fractions. Erf4(6HA)p was found exclusively in the pellet fraction (Fig. 2), as observed previously (37). Na2CO3 and NaCl, which typically release peripheral membrane proteins, fail to extract Erf4(6HA)p (39, 40). Surprisingly, Triton X-100 (1%) and CHAPS (1%) were also unable to release Erf4(6HA)p. Therefore, membrane-associated Erf4(6HA)p exhibits characteristics of an insoluble protein complex. Consistent with this prediction, a relatively high concentration of urea (4.3 M) was able to extract Erf4(6HA)p (Fig. 2). The immunoblots were also probed with antibodies against the plasma membrane ATPase (Pma1p), 3-phosphoglycerate kinase (Pglp1p), and the Ras1 and Ras2 proteins. Pma1p was chosen as an example of a multiple
transmembrane protein that has been shown to exist in a Triton-insoluble fraction in the plasma membrane (41). Pgk1p is a cytosolic protein. Ras2p was released by 1% Triton but not 4.3 M urea as expected for a lipid-anchored membrane protein.

The postnuclear supernatant was also subjected to sucrose gradient fractionation to assess Erf4(6HA)p localization. In the presence of Mg$^{2+}$, Erf4p co-fractionates with Sec61p (ER marker) and Pma1p (plasma membrane marker) (Fig. 3A, upper panel). The addition of 10 mM EDTA allows the plasma membrane and ER to be separated (34). Under these conditions, Erf4p co-fractionates with the ER marker Sec61p (Fig. 3A, lower panel). The ER localization of Erf4(6HA)p was confirmed by indirect immunofluorescence. Both Erf4p and Sec61p exhibit a perinuclear staining pattern (Fig. 3, B and C). The other protein identified from our genetic screen, Erf2p, is also localized to ER in a Triton-insoluble complex (22). This, together with genetic studies showing that erf2 and erf4 mutants have identical phenotypes and the erf2 erf4 double mutant does not exhibit a more severe phenotype than either of the single mutants, suggests that Erf4p and Erf2p may have related functions and perhaps even associate in a complex.

**Evidence for a Direct Interaction between Erf4p and Erf2p**—

The growth defect of the erf2 mutant strain RJY1054 (ras2CSext erf2–8 [YCp52-Ras2]) with a missense mutation erf2I180K can be partially rescued by overexpression of Erf4p, whereas the Erf4p gene cannot suppress an erf2 deletion (Fig. 4A). Increasing the amount of Erf2p in the cell likewise rescues some but not all erf4 mutant alleles. For example, high copy Erf2 partially suppresses strains harboring erf4L204P and erf4S128P, but not erf4V148K (Fig. 4B).

Allele-specific, dosage-dependent suppression can be indicative of a direct protein-protein interaction. Two-hybrid assays were performed to test whether Erf4p and Erf2p do interact. As shown in Fig. 5A, interactions were observed with Erf4p expressed as the bait and Erf2p as the prey as well as with Erf2p bait and Erf4p prey. No interaction was seen between Erf4p and itself or between Erf2p and itself (data not shown). The interaction between Erf4p and Erf2p was confirmed by GST affinity chromatography and FLAG immunoprecipitation (Fig.
Figure 3. Erf4p colocalizes with the endoplasmic reticulum. A, total cell lysates were fractionated by sucrose gradient centrifugation as described under “Experimental Procedures.” Samples were collected from the top (fraction 1) to the bottom (fraction 24) of the gradient, and proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes, and processed for immunoblot with anti-Pma1, anti-Sec61, and anti-HA antibodies. Samples were prepared either in the presence or absence of magnesium (2 mM Mg²⁺, top panel) or EDTA (10 mM EDTA, bottom panel). RJJ266 was transformed with a low copy plasmid expressing Erf4p(6HA) from its own promoter. Cells were fixed, and immunofluorescence was performed using mouse anti-HA IgG to detect Erf2p(6HA)p and rabbit anti-Sec61p IgG to detect endogenous levels of the ER marker Sec61p (C). Antibody complexes were detected using goat anti-mouse IgG-alexa488 conjugate and goat anti-rabbit IgG-rhodamine conjugate.

5B). To begin to map the regions in Erf4p required to interact with Erf2p, we performed co-immunoprecipitation assays from strains expressing FLAG-tagged Erf2p and either wild type or fragments of GST-Erf4p (Fig. 5C). Although the putative leucine zipper is a potential protein-protein interaction domain, truncating Erf4p prior to the leucine zipper (Erf4p(1–166)) had no effect on the ability of Erf4p to interact with Erf2p (Fig. 5C). However, deletion of an additional 30 residues, which removes the weak hydrophobic domain (Erf4p(1–166)), abolishes the Erf2p interaction (Fig. 5C). The same result is obtained if the hydrophobic domain is deleted (Erf4p(Δ167–187)), leaving the rest of the C terminus including the leucine zipper intact (Fig. 5C). Deletion of the hydrophobic domain has no detectable effect on the expression level of Erf4p or the association of Erf4p with the Triton-insoluble complex on the membrane (data not shown).

Loss of Erf4p Function Results in Ras Mislocalization—Galactose-inducible GFP-Ras1p and GFP-Ras2p were constructed to study the subcellular localization of Ras proteins. Both GFP-Ras fusion proteins complement the growth defect of the ras1Δ ras2Δ strain (data not shown). In wild-type cells, the localization of GFP-Ras1 and GFP-Ras2 appears on the rim of the cell indicative of the plasma membrane (22). However, it is now known that Ras proteins are initially targeted to the endoplasmic reticulum prior to translocating to the plasma membrane. The mechanism by which this occurs is not known, but several lines of evidence point to a role for Erf2p and Erf4p. For example, we have shown that Erf2p is involved in the endoplasmic reticulum to plasma membrane translocation of Ras2p. Erf2p and Erf4p exist as a complex on the endoplasmic reticulum membrane and together form the palmitoyltransferase activity that palmitoylates Ras2p on Cys⁶¹⁸ (21). Since palmitoylation correlates with the translocation of Ras2p from the ER to the plasma membrane, we examined whether Erf4p and the interaction between Erf2p and Erf4p are involved in the plasma membrane localization of yeast Ras proteins.

Two well characterized yeast secretory mutants were used to determine whether the classical secretory pathway is required for the plasma membrane localization of GFP-Ras2p. SEC23 encodes the GTPase-activating protein for Sar1p, required for the budding of COPII vesicles from ER (42). SEC14 encodes a phospholipid exchange protein, required for protein transport through the Golgi apparatus (43). As seen in Fig. 6A, the plasma membrane localization of GFP-Ras2p and GFP-Ras1p is not affected by inhibiting the classical secretory pathway by shifting sec23-ts or sec14-ts strains from the permissive (24 °C) to the nonpermissive temperature (37 °C) (Fig. 6A). Deletion of Erf4p alone, as previously observed with Erf2p (22), causes a partial mislocalization of GFP-Ras2p to internal membranes that include the vacuole at 24 or 37 °C (Fig. 6B). If Erf4p is involved in the proposed nonclassical ER to plasma membrane localization pathway, then
deleting ERF4 in the sec-ts strain should lead to a complete mislocalization of GFP-Ras2 when cells are grown at the nonpermissive temperature. This is what was observed (Fig. 6C). Furthermore, it appears that an interaction between Erf2p and Erf4p is required for the trafficking of Ras through this nonclassical ER to plasma membrane translocation pathway. Deletion of the hydrophobic domain does not affect the expression of the mutated Erf4 protein but does diminish the ability of Erf4p to interact with Erf2p. The localization of GFP-Ras1p in a strain expressing Erf4(H9004)167–187 is similar to what is observed in an erf4/H9004 strain (Fig. 7).

Taken together, these results suggest that the translocation of Ras2p from the ER to the plasma membrane does not require the classical secretory pathway as long as Erf2p and Erf4p are present. In the absence of Erf4p, Ras2p requires a functional secretory pathway for plasma membrane localization. The secretory pathway-dependent pathway appears to be less efficient and results in a fraction of the Ras2 protein localized on internal membranes. Since deletion of ERF2 or ERF4 affects the Ras palmitoylation step (22), we propose that Erf2p and Erf4p are components of a palmitoylation-dependent pathway for Ras protein trafficking from the ER to the plasma membrane (Fig. 7).
was examined by confocal microscopy. The localization of GFP-Ras1p and pEG(KT) (vector), pEG(KT)-Erf4, or pEG(KT)-Erf4(Δ167–187) were cultured as described under “Experimental Procedures” and analyzed as described in the legend of Fig. 6. The localization of GFP-Ras1p was examined by confocal microscopy.

**DISCUSSION**

Palmitoylation plays a major role in Ras localization to the plasma membrane as well as Ras signaling (10, 48, 49). However, it is not clear whether palmitoylation is a signal to direct Ras out of the ER or if it is attached to Ras after it reaches the plasma membrane in order to retain it there. These two possibilities are not mutually exclusive. Palmitoyltransferase activities have been detected in both plasma membrane and endomembrane compartments, but efforts to isolate a palmitoyltransferase have been unsuccessful to date (50–52). We previously described a genetic screen designed to identify mutants impaired in the palmitoylation and localization of Ras (22). Mutations in two genes, **ERF2** and **ERF4**, were identified in this screen. Erf2p and Erf4p are associated with the ER membrane and appear to represent the first components of a secretory pathway-independent Ras translocation system. Mutations in the **ERF2** or **ERF4** gene affect not only the localization of Ras (Fig. 7) but also its palmitoylation (22, 37), suggesting that Erf2p and Erf4p are components of the elusive palmitoylation-dependent Ras transport pathway.

Multiple Erf2p homologs have been identified in yeast and other organisms. Previous studies indicate that these homologs (i.e. Psl10p and Ynl326p, etc.) are not involved in Ras function or localization (22). However, a more distant Erf2p-related protein, Akr1p, has been implicated in the subcellular targeting of the type I casein kinase proteins Yck1p and Yck2p (53). Yck1 and Yck2 terminate in a dicysteine motif. Mutation of either cysteine results in a decrease in plasma membrane localization (54). Based on the Yck2p localization defect in **AKR1** mutants and the homology between the cysteine-rich region of Akr1p and Erf2p, it has been suggested that Akr1p may be involved in palmitoylation of Yck2p (53). In fact, Akr1p has recently been shown to palmitoylate Yck2p in an *in vitro* palmitoylation assay (55). We have shown that Akr1p and another yeast DHHC cysteine-rich domain protein encoded by YOL003c can palmitoylate Ras2p, albeit at a lower efficiency than Erf2p.

Despite the ability to readily identify putative Erf2p homologs by sequence homology, we have been unable to identify apparent homologs of Erf4p except in fungal databases (56). This is surprising, since both Erf2p and Erf4p are required for Ras palmitoyltransferase activity (23). It is possible that a functional homolog exists, but the sequence conservation is too low to be detected. Alternatively, other palmitoyltransferases may function as single subunit enzymes. This remains to be determined.

How are Ras proteins translocated from the ER to the plasma membrane? Using sec23-ts and sec14-ts strains, we have shown that the plasma membrane localization of Ras does not require the classical secretory pathway (Fig. 6). The nonclassical pathway for Ras translocation involves Erf4p, because in sec-ts erf4Δ strains plasma membrane localization of Ras is abolished (Fig. 6). However, it seems to be more complicated than this, because deletion of **ERF2**, **ERF4**, or both does not completely abolish plasma membrane localization or the ability of Ras proteins to be palmitoylated (22). We therefore propose a model in which Ras is able to utilize both the classical secretory pathway and an Erf2p/Erf4p-dependent pathway for translocation to the plasma membrane (Fig. 8). Since deletion of **ERF2** and **ERF4** reduces palmitoylation and suppresses the heat shock sensitivity of **RAS2** (Val19) expressing strains (22), we believe that the Erf2p/Erf4p-dependent pathway is the preferred pathway for plasma membrane localization of Ras in yeast. The situation in mammalian cells also appears to be complex. Plasma membrane localization of K-Ras has been shown to be independent of the classical secretory pathway (12). H-Ras, on the other hand, has been reported to require the secretory pathway in order to be localized on the plasma membrane (12). Thus, palmitoylation is not necessary for ER to plasma translocation of Ras in mammalian systems via a nonclassical pathway.

The mechanism by which Erf2p/Erf4p causes the translocation of Ras from the ER to the plasma membrane is currently not clear. It is not known, for example, whether vesicles are involved or if Ras is detached from the membrane by an escort protein and delivered to the plasma membrane. To date, Rab-GDI-like proteins for Ras have not been found. Microtubules have been implicated in K-Ras trafficking in mammalian cells (12, 57, 58). However, neither confocal nor electron microscopic studies support a close proximity between microtubules and K-Ras *in vivo* (12). Since microtubules are involved in multiple aspects of cellular processes, blocking microtubule polymerization may indirectly affect K-Ras localization. Finally, one possibility is that Ras is able to diffuse along an ER membrane network that has been observed to extend from the rough ER proximal to the nucleus all the way to the plasma membrane (59). Additional work will be required to resolve whether these
possibilities hold true for Ras. A better understanding of the molecular mechanisms underlying the subcellular localization of Ras proteins may suggest novel targets for cancer chemotherapeutic drug design.

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