The Erf4 Subunit of the Yeast Ras Palmitoyl Acyltransferase Is Required for Stability of the Acyl-Erf2 Intermediate and Palmitoyl Transfer to a Ras2 Substrate*

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Background: The yeast Ras protein is palmitoylated by a DHHC protein acyltransferase composed of Erf2 and Erf4.

Results: Erf4 affects the stability, autopalmitoylation, and palmitoyl/transferase activity of Erf2.

Conclusion: Erf4 regulates Erf2-dependent palmitoylation of Ras.

Significance: This is the first example of regulation of a DHHC PAT.

Protein S-palmitoylation is a posttranslational modification in which a palmitoyl group is added to a protein via a thioester linkage on cysteine. Palmitoylation is a reversible modification involved in protein membrane targeting, receptor trafficking and signaling, vesicular biogenesis and trafficking, protein aggregation, and protein degradation. An example of the dynamic nature of this modification is the palmitoylation-depalmitoylation cycle that regulates the subcellular trafficking of Ras family GTPases. The Ras protein acyltransferase (PAT) consists of a complex of Erf2-Erf4 and DHHC9-GCP16 in yeast and mammalian cells, respectively. Both subunits are required for PAT activity, but the function of the Erf4 and Gcp16 subunits has not been established. This study elucidates the function of Erf4 and shows that one role of Erf4 is to regulate Erf2 stability through an ubiquitin-mediated pathway. In addition, Erf4 is required for the stable formation of the palmitoyl-Erf2 intermediate, the first step of palmitoyl transfer to protein substrates. In the absence of Erf4, the rate of hydrolysis of the active site palmitoyl thioester intermediate is increased, resulting in reduced palmitoyl transfer to a Ras2 substrate. This is the first demonstration of regulation of a DHHC PAT enzyme by an associated protein.

Protein palmitoylation is involved in the regulation of numerous cellular processes including cell growth and proliferation, protein trafficking, protein turnover, and vesicle fusion (1–4). Defects in protein palmitoylation have been linked to cardiovascular disease, infectious disease, and neurological disorders (5, 6), and more specifically, mutations in the protein acyltransferase (PAT) genes have been linked to colorectal and cervical cancers (7, 8), schizophrenia (9, 10), and X-linked mental retardation (11, 12).

In yeast, the Ras PAT comprises a DHHC protein, Erf2, and a second subunit of unknown function, Erf4. Palmitoylation occurs by a two-step mechanism in which the Cys of the Erf2 DHHC motif undergoes autopalmitoylation to create a thioester-linked palmitoyl intermediate that can either undergo hydrolysis (futile cycle) or transfer the palmitate from the enzyme cysteine to the cysteine of the Ras substrate (13). A similar mechanism has been proposed for other members of the DHHC PAT protein family (14). To date, although Erf4 is required for Erf2-dependent palmitoylation, no specific role has been identified for the Erf4 subunit of the Ras PAT. The mammalian counterpart of Erf2, DHHC9, also co-purifies with a small auxiliary protein, GCP16, and is required for Ras PAT activity (15). Erf4 (also known as Shr5) was originally identified as a suppressor of the lethality resulting from the expression of a hyperactive Ras2 allele (corresponding to oncogenic mutations in mammalian Ras) in yeast (16), suggesting a potential regulatory role for Erf4. Based on sequence homology, putative Erf4 homologs are readily found in other fungal genomes; however, to date, only one metazoan homolog has been identified, GCP16. Although Erf4 and GCP16 associate with membranes, they do not contain the hydrophobic regions one would predict for an integral membrane domain. GCP16 associates with Golgi membranes via a dual palmitoylation site within its coding sequence (17). Erf4, however, has no predictable transmembrane or posttranslational modification consensus sequence motifs and does not require Erf2 for membrane localization (18). One explanation is that Erf4 associates with membranes through an interaction with an integral membrane protein (18). Although it is clear that S-palmitoylation occurs primarily via the DHHC enzyme, the role of the auxiliary subunit remains unclear, and the presence of these auxiliary subunits has posed a long-standing question as to their function in the palmitoylation reaction mechanism. In this report we show that Erf4 functions in stabilizing Erf2 and the autopalmitoylated Erf2 intermediate thioester. In the absence of Erf4, Erf2 undergoes degradation via ubiquitin-mediated mechanisms involving the ER quality control pathway (ERAD). However, stabilization of Erf2 in the absence of Erf4 does not restore palmitoyl transfer-
ase activity, demonstrating that Erf4 has multiple functions. This is the first example of an auxiliary subunit for DHHC PATs that can regulate palmitoylation.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains, Media, and Microbiological Techniques**—The *Saccharomyces cerevisiae* strains used in this study are described in Table 1. Unless stated otherwise, yeast cells were of the S288C genetic background. Yeast cultures were grown in rich medium (yeast extract and peptone (YEP)) or synthetic media. Yeast cultures were grown in the S288C genetic background. Yeast strains lacking components of the quality control systems, e.g. ERAD, were constructed by knock-out of specific genes. Wild type genes were replaced with KanMX- or UB221-linked DNA fragments generated by PCR from genomic DNA isolated from the respective deletion mutant strains (Saccharomyces Genome Deletion Project). Primers to amplify the knock-out region of interest were designed to include 100 bps of genomic DNA sequence upstream and downstream of the start and stop codons, thus providing flanking sequences to permit homologous recombination. Knock-out alleles were validated by PCRSouthern analysis of the respective loci. Primer deoxyribonucleotide sequences are available upon request.

**Plasmid Construction**—Plasmids used throughout this study are shown in Table 2. The sequences of the deoxyoligonucleotide primers used to construct Erf2 alleles are available upon request. The Erf2–6R allele was synthesized de novo (Bio Basics, Inc, Amherst, NY). pUB221 was a gift from Daniel Finley, Harvard University, Cambridge, MA (21). pESC-TrpPA4:FLAG (B1165) was a gift from Maurine Linder, Cornell University, Ithaca, NY. B1821 was constructed by inserting a PCR fragment containing the terminal 174 bps of ERF2 (B1119 was used as the template) into B1164 using ligase-independent cloning. B1822 was constructed in a similar manner as B1821 except that B1835 (pUC57:ERF2–6R) was used as the PCR template. B1836 was constructed using pEG(KG) as the vector backbone. The gene for maltose-binding protein (MBP) was amplified by PCR using pMAL (New England Biolabs, Inc, Ipswich, MA) as the template. This resulting product was used to replace the GST gene in pEG(KG) (ligase-independent cloning) to create the final construct. Plasmids B1414 (18), B1302 (22), B924 (18), B1119 (23), B1258 (13), B1259 (13), B1250 (13), B529 (24), B374 (24), and B322 (25) have been described previously. B1825 (pESC-LeuFLAG:ERF2ΔC) was made by inserting the BglII-PacI fragment of B1835 into similarly digested pESC-Leu. B1826 (pESC-LeuFLAG:ERF2–6R) was made by inserting the BglII-PacI fragment of B1835 into pESC-Leu. 6XHis-ERF2 alleles were constructed using the QuickChange II site-directed mutagenesis reaction.
mutagenesis kit (Agilent Technologies, Santa Clara, CA) per the manufacturer's instructions. Isolates produced from the mutagenesis protocol were sequenced to confirm the ERF2 allele changes. Plasmids were rescued (26), and the DNA was sequenced to confirm the presence of the mutations (GeneWiz, South Plainfield, NJ).

**Protein Expression and Purification**—Overexpression of Erf2 and Erf4 proteins was performed essentially as in Mitchell et al. (13). Strain RY1827 was co-transformed with pESC-Leu6×HIS:ESC-2(FLAG)ERF4 and B322 (pMA210) and grown in SC (Leu−His−) medium containing 2% (v/v) ethanol, 2% (v/v) glycerol at 30 °C. The cultures were grown to 2 × 10^7 cells/ml and then induced by adding galactose to a final concentration of 0.8% DDM, 5% glycerol, and 250 mM imidazole. The eluted samples was washed once with Solution W (50 mM Tris-HCl, pH 8.5, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM protease inhibitor mixture, 8 μl/ml saturated PMSF), and the cells were lysed by vortexing using glass beads (400–600 mesh, Sigma) for 40 min with 1.5-min pulses. The resulting extract was spun at 3000 × g for 15 min to remove cellular debris and unbroken cells followed by a crude membrane fraction (P100) by centrifugation (100,000 × g) for 1.5 h at 4 °C with a Beckman 50.2 Ti rotor. The supernatant was discarded, and the pellet was resuspended in Tris-buffered saline, pH 7.4, with the aid of a Dounce homogenizer. The resulting extract was adjusted to a final concentration of 0.8% dodecylmaltoside (DDM), 500 mM NaCl, and 1 mM β-mercaptoethanol (β-ME). To solubilize the membranes, the extract was incubated at 4 °C (1.5 h). To aid in purification, urea and imidazole were added to a final concentration of 2.4M and 1 mM, respectively. Insoluble material was then removed by centrifugation (10,000 × g) for 15 min at 4 °C. The supernatant was incubated with Ni-NTA resin at 4 °C for 2 h with mild agitation. The extract was once again centrifuged at 13,000 × g for 30 min to remove the insoluble fraction. The soluble fraction was incubated with pre-equilibrated amylose resin (New England Biolabs) for 1 h. The resin was washed 3 times with 1× PBS + 1% Triton X-100 and eluted from the resin using 1× PBS + 1% Triton X-100 + 10 mM maltose. The resulting eluent was buffer exchanged to remove the maltose and concentrated.

**Erf2 Stability Assays**—In the case of the GAL promoter-driven Erf2 assays, yeast cells were seeded at an A_600 of 0.1 in 200 ml of synthetic medium supplemented with 2% glycerol and grown to an optical density of 1.0 at 30 °C with shaking. At this time, galactose was added to a final concentration of 2%, and the cultures were incubated at 30 °C with shaking for 3 h. After the incubation, glucose was added to a final concentration of 4% along with cycloheximide (25 μg/ml in DMSO), and 20-ml (20 A_600) volumes were removed at various times and placed into chilled tubes containing 20 mM NaN_3 (final concentration) to stop growth and metabolism. In the case of strains expressing ERF2:13×MYC, cultures were seeded at an A_600 of 0.1 in synthetic medium supplemented with 2% glucose and grown at 30 °C with shaking to an A_600 of 1.0. Cycloheximide (25 μg/ml in DMSO) was added, and 20-ml volumes were removed at various times and placed into chilled tubes containing NaN_3 (20 mM final). For both procedures, after all the time points were collected, cells were harvested by centrifugation (3500 × g), and the cell pellets were washed with Tris-buffered saline, pH 7.4, containing 20 mM NaN_3. The cells were broken in Thorner buffer (40 mM Tris-HCl, pH 6.8, 5% SDS, 8 mM Urea, 100 μM EDTA, 5 mM β-mercaptoethanol, and 0.004% bromphenol blue) plus glass beads. Samples were analyzed by Western blot. FLAG-tagged proteins were transferred to a nitrocellulose membrane and visualized by ECL (Pierce) using mouse anti-FLAG M2 antibody (Sigma) as primary antibody and anti-mouse IgG horseradish peroxidase conjugate as secondary antibody.

**Coupled PAT Assay**—The coupled fluorescence assay was performed as in Mitchell et al. (13). Briefly, the production of NADH was monitored with a Biotek Mx fluorimeter (Biotek, Winooski, VT) using 340-nm excitation/465-nm emission. The 200-μl reaction contained 2 mM 2-oxoglutarate (α-ketoglutaric acid), 0.25 mM NAD^+, 0.2 mM thiamine pyrophosphate, 0.4–1 μg of purified 6×His-Erf2-ERF4 complex, 1 mM EDTA, 1 mM dithiothreitol, 32 milliunits of 2-oxoglutarate dehydrogenase (α-ketoglutarate dehydrogenase), 50 mM sodium phosphate, pH 6.8. The reaction was initiated by the addition of different concentrations of palmitoyl-CoA and monitored for 30 min at 30 °C. The first 10 min of the reaction was analyzed to determine the initial rates of CoASH release. Autopalmitolya-
Regulation of Protein Acyl Transferases

U287 • N41 •

tion activity was determined from a standard curve of NADH production as a function of CoASH concentration. In these reactions CoASH was added to the standard PAT reaction mixture (without Erf2-Erf4 complex or palmitoyl-CoA), and the reaction was allowed to proceed to equilibrium (10 s) before fluorescence was measured (excitation 340 nm/emission 465 nm). Assays using the catalytically dead C203S derivatives of the Erf2 complexes were performed, and the values were subtracted from the rates obtained for the respective Erf2 complexes. The values obtained for the varying concentrations of palmitoyl-CoA were fitted using Graphpad Prism software v4.0.

Bodipy C12:0 Transfer Assay—Bodipy C12:0-CoA (40 μM final) was added to a 25-μL reaction containing ~2.0 μg of enzyme (FLAG-Erf2-Erf4) bound to anti-FLAG-tagged agarose and 100 pmol of purified MBP:mCherry:Ras2CT35 (wt) in 0.05 mM sodium phosphate buffer, pH 7.4, and incubated at 30 °C. After 30 min, 5× non-reducing loading buffer was added to each sample. Each reaction was heated at 65 °C for 3 min and then subjected to SDS-PAGE (12%). The gel was washed 3 times in double distilled H2O and visualized on the Typhoon 9410 Variable Mode Imager (GE Healthcare) for Bodipy fluorescence (excitation 485 nm/emission 528 nm) to visualize transfer of the Bodipy signal to MBP:mCherry:Ras2CT35 and for mCherry fluorescence (excitation 520 nm/emission 610 nm) to determine the amount of MBP:mCherry:Ras2CT35 loaded per lane. The amount of FLAG-Erf2 was determined empirically using SDS-PAGE under reducing conditions.

Complementation Assay—The in vivo function of the mutants along with the wild type protein was investigated using our previously described complementation assay (27). Briefly, in this assay, cells contain a defective allele of RAS2 that is balanced by an episomal copy of RAS2 linked to URA3. Under these conditions, the loss of the Erf2 gene is permissible as long as the cell maintains the RAS2-URA3-based episome. This is detected by the ability or inability of the strain to grow on medium supplemented with 5′-fluoroorotic acid (FOA) (28). Cells carrying Erf2 alleles were transformed into Rj11888 and plated on synthetic medium containing glucose and lacking leucine. Colonies were inoculated into liquid synthetic medium containing glucose or raffinose (both lacking tryptophan) and grown to mid-log phase, cycloheximide was added (ρ₀), and Erf2 levels were analyzed using an anti-Myc epitope tag antibody (Fig. 1A). The amount of phosphoglycerate kinase (PGK) was used for normalization of the samples. In the Erf4 wild type strain, the half-life of Erf2 was 153 min, whereas in the strain lacking Erf4 (erf4Δ), the half-life of Erf2 was reduced to 53 min (Fig. 1B).

One possible explanation for the instability of Erf2 in the absence of Erf4 is that the loss of the Erf4 interaction may uncover a domain that is linked to Erf2 instability. To map the region of Erf2 that may contribute to its degradation, we constructed Erf2 mutants lacking the first 119 amino acids (N-terminal deletion, Erf2ΔN) or the final 58 amino acids (C-terminal deletion, Erf2ΔC) that were under the control of the GAL1,10 promoter (13). Deletion of the N terminus had no effect on increasing the half-life of Erf2 in the absence of Erf4 (22 min) when compared with wild type Erf2 (25 min) under the same conditions. However, when the C-terminal 58 amino acids were truncated, the half-life of Erf2 increased to 361 min, ~2-fold that of wild type Erf2 in the presence of Erf4F (169 min). A possible explanation for the increased stability of Erf2ΔC is that it may not be properly localized and the stability may reflect nonnative subcellular localization where there is a different molecular degradation mechanism. However, like the WT GFP: Erf2-Erf4 complex, the bulk of GFP:Erf2ΔC, in the absence of Erf4, localized to the perinuclear ER.3

Ubiquitin-dependent Clearance of Erf2 by the ERAD System—The Erf2-Erf4 complex localizes to the ER where it palmitoylates Ras (18, 27, 30). We speculated that ER quality control mechanisms would be involved in Erf2 turnover and, therefore, examined Erf2 for the presence of covalently attached ubiquitin (31). FLAG:Erf2 was co-expressed with 6×Hisubiquitin (pUb221) in a strain lacking Erf4 (21). The cell extract was enriched for ubiquitylated proteins using Ni-NTA-agarose and immuno-

3 K. Ishizuka and R. J. Deschenes, unpublished results.
blotted for Erf2 using anti-FLAG M2 antibodies (Fig. 2A). The signal was specific for both FLAG:Erf2 and 6×His:Ub and appeared as a smear above the band representing FLAG:Erf2 alone, presumably due to heterogeneous polyubiquitylated FLAG:Erf2 (Fig. 2A). These data are consistent with the role of the quality control system in degrading Erf2 molecules that are not in complex with Erf4.

To investigate whether the ERAD pathway is involved in Erf2 turnover, the steady state half-life of Erf2 was measured in wild type yeast strains or harboring a deletion in key ERAD genes (Fig. 2B). The ERAD pathway has been defined by mutation in genes encoding proteins that monitor cytosolic-facing, ER lumen-facing, or general features of ER membrane proteins (32–34). Erf2 is predicted to be an integral membrane protein with four transmembrane-spanning segments, short ER lumen (32–34). Erf2 is predicted to be an integral membrane protein in genes encoding proteins that monitor cytosolic-facing, ER lumen-facing, or general features of ER membrane proteins.

Strains harboring stabilized Erf2 still require Erf4, suggesting an additional function for Erf4—The loss of a subset of the ERAD components increased the stability of Erf2 in the absence of Erf4. We, therefore, asked whether, under these conditions, there was a restoration of Erf2 function. In other words, was Erf4 required for Ras palmitoylation beyond its role in Erf2 localization?
stabilization. We utilized the plasmid shuffle assay that was originally used to identify ERF2 and ERF4 (27) using RJY1620 (18) deleted for the ERAD components (above). This strain has the palmitoylation-dependent Ras2 (RAS2 CS-Ext) allele at the RAS2 locus and harbors a plasmid-expressing wild type RAS2 that can be lost by asymmetric segregation if Ras2 CS-Ext is palmitoylated. We observed that only the presence of wild type ERF4 would allow the loss of the RAS2-based plasmid on 5′-FOA (Fig. 4A). Although some of the ERAD mutants could increase the stability of Erf2 in the absence of Erf4 (Fig. 2B), these mutants were unable to suppress the loss of ERF4 under these conditions (Fig. 4A). We also examined the ability of stabilized Erf2–6R to suppress the loss of ERF4. As with the loss of the ERAD components, expression of FLAG:ERF2, FLAG:ERF2ΔC, or FLAG:ERF2–6R was unable to suppress the growth defect of strains lacking Erf4 (Fig. 4B). Together, these in vivo data demonstrate that the role Erf4 plays in palmitoyl transferase activity extends beyond solely stabilizing Erf2. We were sur-

FIGURE 2. Degradation of Erf2 involves polyubiquitinylation and the ERAD system. A, upper panel, to determine if Erf2 undergoes ubiquitinylation, extracts from diploid erf4/C strains expressing FLAG:ERF2 with and without pUb221 (6×His:ubiquitin) or FLAG:ERF2–6R with and without pUb221 were treated with 6M guanidine-hydrochloride (to denature and dissociate all non-covalently associated proteins), and ubiquitinated proteins were isolated using Ni-NTA-agarose, separated by SDS-PAGE, and immunoblotted with anti-FLAG antibodies (to detect Erf2). Strains expressing both plasmids produced polyubiquitin-conjugated Erf2 molecules as seen by a smear larger than the apparent molecular weight of FLAG:Erf2. Lower panel, whole cell extracts from the strains used in the upper panel were separated by SDS-PAGE and immunoblotted with anti-FLAG antibody to demonstrate the presence of FLAG:Erf2. B, the half-life of 13xMyc:Erf2 in the absence of Erf4 is increased by deleting the ER quality control components. The bar graph shows the data from two independent experiments (black and gray bars) for the half-life of Erf2 in isogenic strains erf4, yos9 erf4, rpm10 erf4, hrd1 erf4, ubc7 erf4, and doa10 erf4 compared with the wild type (ERF4) strain.

FIGURE 3. The C-terminal 58 amino acids of Erf2 are sufficient to promote degradation. A, shown is a schematic representation of ER localized acyltransferase, Pfa4, with the C-terminal FLAG epitope and Erf2 58 amino acid additions. The amino acid sequence below the schematic compares the wild type Erf2 C-terminal 58 amino acids with that of Erf2–6R in which the six lysines are mutated to arginines (asterisks). B, representative Western analysis comparing the amounts of Pfa4:FLAG, Pfa4:FLAG:ERF2(C58), Pfa4:FLAG:ERF2–6R, FLAG:Erf2, and FLAG:Erf2–6R at the indicated times after cycloheximide (25 μg/ml) addition probed with antibodies to the FLAG epitope (Sigma). C, shown is a comparison of the half-lives of Pfa4:FLAG, Pfa4:FLAG:ERF2(C58), Pfa4:FLAG:ERF2–6R, FLAG:Erf2, and FLAG:Erf2–6R. The bar graph shows the data from two independent experiments (black and gray bars) determining the half-life of the indicated fusion proteins.
FIGURE 4. Stabilized Erf2 cannot suppress the loss of ERF4. Shown is a functional plasmid shuffle assay to determine the ability different Erf2 stabilizing conditions to suppress the loss of ERF4. A, the genes for ERAD components were deleted from RY1620 and plated on synthetic medium lacking uracil to demonstrate the presence of the sectoring plasmid. These strains, harboring pRS314 (TRP1) or B1414 (pRS314ERF4), were replicated to synthetic medium lacking tryptophan and supplemented with 5′-FOA to select for those strains capable of losing the UR3 linked RAS2 episome. B, shown is a series dilution of strain RY1888 cultures harboring plasmids expressing the Erf2 alleles with and without ERF4. Cells were spotted in 10-fold dilutions (10⁴ initial cfu) on medium lacking leucine (left panel) and medium lacking leucine supplemented with 5′-FOA (right panel) and incubated for 4 days at 30 °C.

prised to observe that FLAG:ERF2ΔC-ERF4 could also suppress the erf2Δ erf4Δ phenotype of strain RY1888, albeit at a low level (~1/1000 that of FLAG:ERF2-ERF4). This suppression was dependent on the FLAG:ERF2ΔC-ERF4 plasmid (data not shown).

Erf4 Is Required for Stable Formation of the Erf2-Palmitoyl Intermediate—The Erf2-Erf4 enzyme reaction proceeds via a two-step mechanism (13). In the first step, Erf2 is palmitoyl-oylated using palmitoyl-CoA as substrate, releasing reduced CoASH (1, 4). The second step of the reaction transfers the palmitate from Erf2 to the protein substrate. The palmitoylation reaction can be assessed by two assays. In the first, the steady state amount of palmitoylated Erf2 is determined by performing the palmitoylation reaction using a tagged palmitoyl-CoA probe, and the reaction products were separated by non-reducing SDS-PAGE (23). The limitation of this assay is that one cannot differentiate between an enzyme that can undergo palmitoylation with rapid hydrolysis of the thioester linkage and an enzyme that does not get palmitoylated. In the second assay, the rate of palmitoyl-CoA reacted is monitored by measuring the production rate of CoASH, a product of the reaction (13). This assay takes into account the formation and subsequent hydrolysis of the palmitoyl-Erf2 thioester intermediate. Together, these assays provide an accurate measure of palmitoylation and thioester hydrolysis. FLAG:ERF2, FLAG:ERF2–6R, and FLAG:ERF2ΔC were expressed in yeast cells (RJ1828) with and without ERF4 (GST: ERF4). The PAT complexes were partially purified, and the amount of enzyme was determined by SDS-PAGE using a known standard. To determine the steady state amount of acyl-Erf2 intermediate, we reacted the isolated FLAG:Erf2 molecules with Bodipy C12:0-CoA, an analog of palmitoyl-CoA, and the products of the reaction were separated by non-reducing SDS-PAGE (Fig. 5A). The steady state amount of Bodipy C12-Erf2 was greater in the full-length stabilized mutant (Erf2–6R-ERF4) when compared with wild type Erf2-Erf4. When Erf4 is absent, the steady state amount of Bodipy C12-Erf2–6R intermediate drops more than 2 orders of magnitude. A similar phenomenon was observed for wild type Erf2 in the absence of Erf4. Interestingly, the steady state amount of Erf2ΔC was independent of Erf4, albeit at ~30% that of the level of wild type Erf2-Erf4, suggesting that loss of the C-terminal 58 amino acids of Erf2 may influence its association with Erf4.

On the surface these data seem to argue for a diminution of autopalmitoylation activity for Erf2 in the absence of Erf4. However, when the rate of CoASH production is measured after the steady state of the reaction is reached, we observed an increase in autopalmitoylation and the palmitoyl-Erf2 intermediate thioester hydrolysis rate for Erf2 (Fig. 5B, closed circles) (3.3-fold) and Erf2–6R (Fig. 5B, closed squares) (2.4-fold) in the absence of Erf4 (Table 3) as determined by an increase in the VMAX of the respective reactions. We also observed the greatest CoASH production rates using the Erf2ΔC (Fig. 5B, open triangles) and Erf2ΔC-Erf4 (Fig. 5B, closed triangles) proteins implying that autopalmitoylation and thioester hydrolysis are greater for these mutants. To ensure that the activity signal we were detecting is due to the formation and hydrolysis of the palmitoyl-Erf2 thioester intermediate (located at residue Cys-203 of Erf2), we mutated the codon for Cys-203 to a serine codon (C203S) for all the Erf2 alleles (with and without ERF4) and repeated the experiment, subtracting the rates obtained for the C203S proteins from the corresponding Erf2-dependent activities. These data demonstrate that (a) the Erf2 protein is capable of forming and hydrolyzing the palmitoyl-Erf2 thioester intermediate in the absence of Erf4 and (b) in the cases of Erf2 and Erf2–6R, the presence of Erf4 decreases the VMAX of the reaction, suggesting that Erf4 has the potential for regulating autopalmitoylation/hydrolysis, possibly by controlling access to the active site.

Erf4 Is Required for Palmitoyl Transfer to Ras—Finally, we determined the ability of Erf2 to transfer the fluorescent palmitoyl-CoA analog, Bodipy C12:0-CoA, to a Ras2 substrate (Fig. 6). In these experiments Erf2 with or without Erf4 were affinity to beads through antibodies to the FLAG epitope. MBP:mCherry::Ras2CT35 (100 pmol) was added, and the reaction was initiated by the addition of Bodipy C12:0-CoA (1.2 nmol). Normalizations for the amount of Erf2 protein added to the reactions are shown in the lower panel of Fig. 5A. The number of moles of Bodipy C12:0-CoA transferred was determined empirically using a standard curve (data not shown). As expected, more Bodipy C12 was transferred when Erf4 was present with Erf2 and Erf2–6R. Previously, we demonstrated that although Erf2-Erf4 autopalmitoylation proceeds with burst kinetics, the transfer of palmitate to a Ras2 substrate proceeds by what appears to be first order kinetics (13) in that the...
reaction signal is linear with respect to time, does not demonstrate a burst of activity, and appears dependent on the concentration of Ras2. We determined the amount of Bodipy C12 transferred to Ras2 to be 4.8 pmol (1739 pmol/min/µmol of MBP:mCherry:Ras2CT35) for Erf2-Erf4 and 4.0 pmol (1515 pmol/min/µmol of MBP:mCherry:Ras2CT35) for Erf2–6R-Erf4. In comparison, a value of 940 pmol/min/µmol of GST:Ras2 was obtained using [3H]palmitoyl-CoA and GST:Ras2 as substrates (23). We were intrigued to observe a small, yet detectable amount of Bodipy C12 transferred to Ras2 using Erf2 C-Erf4 of 0.6 pmol (182 pmol/min/µmol of MBP:mCherry:Ras2CT35). One could imagine that the relatively small amount transferred to the Ras2 substrate may explain the detectable, yet reduced viability we observed for our growth assay (Fig. 4B) for Erf2 C-Erf4. We also detected amounts of

FIGURE 5. Erf4 dependence of Erf2 autopalmitoylation. A, in vitro autopalmitoylation reactions using Bodipy C12:0-CoA as the acyl donor are shown. Top panel, in vitro autopalmitoylation reactions were separated using SDS-PAGE under non-reducing conditions, and the fluorescence was visualized using excitation 488-nm/emission 520-nm filters (Typhoon, GE). The middle panel of A shows a representative Western blot (WB) analysis used to quantify the amount of Erf2 and Erf2 mutants. The bar graph shows the amount of autopalmitoylation normalized to the amount of Erf2 protein present in each sample (bottom panel). The asterisk denotes cross-reactivity of anti-FLAG with reduced small chain IgG from the antibody coated agarose beads. B, shown is post-steady state autopalmitoylation and hydrolysis fluorescence assay that couples the production of CoASH, a product of the autopalmitoylation reaction, with the reduction of NAD\(^+\) (NADH) using α-ketoglutarate dehydrogenase. Assays were performed varying the amount of palmitoyl-CoA. The background values were determined by performing the assays using the analogous catalytically impaired Erf2 enzymes (Erf2 C203S), and those values were subtracted from the values obtained using the active Erf2 enzymes. The data represent Erf2ΔC (open triangle), Erf2ΔC-Erf4 (closed triangle), Erf2-Erf4 (open circle), Erf2 (closed circle), Erf2–6R-Erf4 (open square), and Erf2–6R (closed square). The data were fit using Prizm software, n = 4. K\(_{\text{m}}\), V\(_{\text{M}}\)\(\text{MAX}\), and k\(_{\text{cat}}/K\(_{\text{m}}\) values are shown in Table 3.

| Erf2 complex   | K\(_{\text{m}}\) (µM) | V\(_{\text{M}}\)\(\text{MAX}\) (pmol/min/µg) | k\(_{\text{cat}}/K\(_{\text{m}}\) (min\(^{-1}\)µmol\(^{-1}\)) |
|---------------|------------------------|--------------------------------------------|--------------------------------------------------|
| Erf2-Erf4     | 43 ± 8                 | 43 ± 3                                     | 66,667                                          |
| Erf2          | 20 ± 1                 | 143 ± 11                                   | 476,667                                         |
| Erf2ΔC-Erf4   | 41 ± 4                 | 460 ± 21                                   | 659,971                                         |
| Erf2ΔC        | 29 ± 8                 | 385 ± 54                                   | 780,933                                         |
| Erf2–6R-Erf4  | 20 ± 3                 | 52 ± 6                                     | 173,333                                         |
| Erf2–6R       | 16 ± 2                 | 125 ± 4                                    | 520,833                                         |

TABLE 3

Erf2 complex autopalmitoylation/thioester hydrolysis activities

transferred to Ras2 to be ~4.8 pmol (1739 pmol/min/µmol of MBP:mCherry:Ras2CT35) for Erf2-Erf4 and 4.0 pmol (1515 pmol/min/µmol of MBP:mCherry:Ras2CT35) for Erf2–6R-Erf4. In comparison, a value of 940 pmol/min/µmol of GST:Ras2 was obtained using [3H]palmitoyl-CoA and GST:Ras2 as substrates (23). We were intrigued to observe a small, yet detectable amount of Bodipy C12 transferred to Ras2 using Erf2ΔC-Erf4 of 0.6 pmol (182 pmol/min/µmol of MBP:mCherry:Ras2CT35). One could imagine that the relatively small amount transferred to the Ras2 substrate may explain the detectable, yet reduced viability we observed for our growth assay (Fig. 4B) for Erf2ΔC-Erf4. We also detected amounts of
transfer for Erf2ΔC and Erf2–6R of 0.4 pmol (150 and 152 pmol/min/μmol of MBP:mCherry:Ras2CT35, respectively) in the absence of Erf4 that were greater than with Erf2 (0.2 pmol; 88 pmol/min/μmol of MBP:mCherry:Ras2CT35) alone. The amount of background fluorescence, presumably non-catalytic palmitoylation, observed for the Ras2 substrate alone was well below the detection limit for the assay and was estimated based on background subtraction to be less than 5% of the Erf2-Erf4-dependent transfer signal. Although the MBP:mCherry:Ras2CT35 protein migrates as a doublet, we detected palmitate transfer in only the top band of the doublet, suggesting that the doublet is formed from proteolysis of the CAAX box tail of the full-length protein. In addition to the palmitoylated Ras2 gel band, we also observe nonspecific bands that appear to be contaminants from the Bodipy C12:0-CoA synthesis.

DISCUSSION

The Ras PAT, Erf2-Erf4, is a member of a large family of enzymes that has at least seven members in fungi and >20 members in higher eukaryotes (1). Like the vast majority of members of the PAT family, the Erf2 component of this complex contains the canonical DHHC motif and is the subunit involved in the formation of the palmitoyl-enzyme intermediate (13, 23, 41, 42). However, Erf2 requires an accessory subunit, Erf4, for palmitoyl transfer activity (23, 27). Erf4/SHR5 has been identified twice in genetic screens aimed at identifying regulators of the Ras pathway in *S. cerevisiae* (16). This heterodimer stoichiometry is also observed for the mammalian homologue of Erf2, DHHC9, and its accessory subunit, GCP16 (15). The identification of GCP16, the accessory subunit of mammalian Ras PAT, DHHC9, was based on the primary amino acid sequences of the fungal Erf4 family. Currently, only the Ras PAT enzymes have been shown to require an accessory subunit in addition to the DHHC subunit. In previous reports, we demonstrated that (a) Erf2 and Erf4 interact (18, 27), (b) the Erf2-Erf4 (and DHHC9-GCP16) interaction is required for the enzymatic activity of the Ras protein acyltransferase (15, 23), and (c) the Erf2-Erf4 protein acyltransferase complex transfers palmitate from a donor (palmitoyl-CoA) to a protein substrate (Ras2) using a two-step reaction mechanism (13). Although the reaction mechanism has been determined, the contribution of the individual subunits to palmitate transfer has not been addressed before this study.

Here we show that Erf4 regulates the autopalmitoylation state of the enzyme by stabilizing the palmitoyl-Erf2 interme-
Regulation of Protein Acyl Transferases

diate and also is required for the second transfer step of the reaction. Erf4, therefore, potentially plays a role in transfer catalysis, substrate recognition, or both. In the past the role of Erf4 in palmitoylation has been clouded by the inability to accurately measure the palmitoylation activity of Erf2 in the absence of Erf4 (22, 23). Steady state amounts of Erf2 are decreased ~40-fold in the absence of Erf4 in vivo, an observation that implies Erf4 stabilizes or impedes the degradation of Erf2 in the cell. We show this to be the case. The half-life of Erf2 is reduced from 153 to 50 min in the absence of Erf4. To determine if specific amino acid sequences or domains are involved in Erf2 degradation, we parsed Erf2 into three domains; the N-terminal (amino acids 1–119), DHHC (amino acids 120–300), and C-terminal (amino acids 301–359). The DHHC domain serves as the catalytic core of the PAT, able to perform the first step of palmitoylation, autopalmitoylation, but not sufficient to have PAT transfer activity by itself (13). Unlike the DHHC domain, N- and C-terminal domains of Erf2 do not share significant homology with other PATs or any known proteins (1). Deletion of the N-terminal domain had no effect on stabilizing Erf2 in the absence of Erf4. However, deletion of the C-terminal domain in the absence of Erf4 increased the half-life of Erf2 to 2-fold greater than that of the wild type enzyme in the presence of Erf4. The degradation of Erf2 is an ERAD-mediated event resulting in polyubiquitinylation of, and facilitated by the C-terminal 58 amino acids of Erf2. This was confirmed by creating an Erf2 C-terminal fusion of this sequence with another endoplasmic reticulum localized PAT, Pfa4 (Pfa4:FLAG:Erf2 C58). The addition of the C-terminal domain decreased the half-life of Pfa4 by 75%. Stabilizing Erf2 either by removing the C-terminal 58 amino acids or changing the 6 lysines within that domain to arginines, however, does not suppress the loss of Erf4 in vivo. Additionally, deletion of the 58 C-terminal amino acids has considerable influence on the palmitoyl transfer activity of the enzyme in vitro. Finally, we observed that Erf4 has a negative regulatory effect on the post steady state autopalmitoylation/hydrolysis activities of Erf2. One possible molecular explanation is that Erf4 binds Erf2 through the C-terminal domain. To date, we have been unable to detect an interaction between Erf4 and the Erf2 C-terminal domain (data not shown). Alternatively, the association of Erf4 with Erf2 could cause a conformational change that buries or masks the C-terminal domain in some way that does not involve association with Erf4, making it inaccessible to the degradation machinery. It is difficult to say at this point which model reflects reality. However, it is clear that the association of Erf4 with Erf2 regulates the amount of Erf2 and, therefore, controls the amount of palmitate transferred to Ras substrates. Taken together, these data support the notion that Erf4, although important for stabilizing Erf2, is required for palmitate transfer to protein substrates and is consistent with a role in catalysis and/or substrate recognition.

We were intrigued to observe that Erf4 had an inhibitory effect on the rate of autopalmitoylation/hydrolysis cycling. Previously, we had formulated the hypothesis that Erf2, in the absence of Erf4, was unable to perform either step of the palmitoylation reaction based on the inability to detect [3H]palmitoyl-labeled Erf2 by autoradiography (23). However, we developed an assay (13) that monitors the production of CoASH, one of the products of autopalmitoylation, by coupling its formation to the reduction of NAD⁺ using a-ketoglutarate dehydrogenase as the catalyst. Although this assay does not measure the pre-steady state burst kinetics, it does monitor the post-steady state kinetics of the autopalmitoylation and hydrolysis reactions, which can give insights into the enzyme molecular mechanism. Based on the result of this assay and the palmitoyl transfer assay, three conclusions can be drawn. First, the absence of Erf4 increases the $V_{\text{MAX}}$ of the autopalmitoylation reaction for wild type Erf2 and Erf2–6R by 3.3- and 2.4-fold, respectively, while having little effect on the $K_m$. One possible explanation is that Erf4 protects the active site intermediate thioester from hydrolysis, potentially by limiting access to water. Secondly, deletion of the C-terminal 58 amino acids has a greater effect on increasing the autopalmitoylation reaction $V_{\text{MAX}}$ and appears to be independent of the presence of Erf4, implying that the C terminus of Erf2 also participates in protecting the active site. This may occur directly by forming a domain capable of shielding the active site or indirectly by affecting the overall folding of the enzyme. Finally, Erf4 is required for the transfer of the palmitoyl group from Erf2 to the protein substrate. However, it remains to be determined whether Erf4 participates in substrate recognition, transfer catalysis, or both.

We have demonstrated that Erf4 plays a role not only in stabilizing Erf2 but also in promoting the transfer of palmitate from the palmitoyl-enzyme intermediate to the protein substrate. Can this observation be extended to the other PATs? DHHC-mediated protein palmitoylation occurs in two steps: autopalmitoylation of the DHHC molecule to form a palmitoyl-enzyme intermediate, which appears to be a universal activity (4, 42, 43), and transfer of the modifying palmitate to a protein substrate. Our data highlight a subset of functions for the Erf4 subunit. First, Erf4 protects Erf2 from ubiquitin-mediated degradation. Although the mechanism is not immediately clear, one possible explanation is that Erf4 is involved in the proper conformation of Erf2 within the endoplasmic reticulum. Aside from its effect on Erf2 stability, the surprising aspect of Erf4 function is its effect on autopalmitoylation. The loss of Erf4 does not abolish autopalmitoylation, as would be the case if residues of Erf4 participated in autopalmitoylation catalysis. Recently, we demonstrated that autopalmitoylation of the Erf2 active site, which occurs with burst kinetics, could be followed by hydrolysis of the palmitoyl-Erf2 intermediate thioester linkage in the absence of a protein substrate (13). It appears that loss of Erf4 may increase the hydrolysis rate of the thioester of the intermediate, causing the enzyme to undergo rapid cycles of autopalmitoylation and hydrolysis. Erf4, therefore, acts to limit the access of water to the active site. This observation is also true for the effect of GCP16 on DHHC9. An increase in the hydrolysis rate would come at the expense of the steady state amount of palmitoyl-enzyme intermediate and ultimately, a decrease in the amount of palmitate that gets transferred to the protein substrate. The juxtaposition of the DHHC domain with the hydrophobic milieu of the membrane, hypothesized for all DHHC enzymes (35), may be a mechanism for limiting water

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4 D. A. Mitchell and R. J. Deschenes, unpublished results.
molecules from invading the active site. In addition, Erf4 is required for the transfer of palmitate to the protein substrate. One possibility is that Erf4 is involved in substrate recognition. Another, non-mutually exclusive hypothesis is that residues of Erf4 are required for catalysis of the palmitoyl transfer step. It is evident, however, that the mechanism of autopalmitoylation of DHHC enzymes requires shielding the active site from water, a job that may be performed by residues/domains of the DHHC molecule or by another accessory protein subunit. It is, therefore, conceivable that accessory subunits exist for many, if not all, DHHC enzymes and that their identification has gone undetected. Taken together, these data provide an initial elucidation of the molecular mechanism underlying the role of accessory proteins in protein palmitoyl transfer, and we are now beginning to address some of the questions directed at the activity and substrate recognition of palmitoyltransferases as a whole.

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