HHS Public Access

Author manuscript

Yeast. Author manuscript; available in PMC 2018 March 27.

Published in final edited form as:

Yeast. 2010 June ; 27(6): 327–343. doi:10.1002/yea.1756.

Chemical inhibition of CaaX protease activity disrupts yeast Ras localization

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Abstract

Proteins possessing a C-terminal CaaX motif, such as the Ras GTPases, undergo extensive post-translational modification that includes attachment of an isoprenoid lipid, proteolytic processing and carboxylmethylation. Inhibition of the enzymes involved in these processes is considered a cancer-therapeutic strategy. We previously identified nine *in vitro* inhibitors of the yeast CaaX protease Rce1p in a chemical library screen (Manandhar *et al.*, 2007). Here, we demonstrate that these agents disrupt the normal plasma membrane distribution of yeast GFP–Ras reporters in a manner that pharmacologically phenocopies effects observed upon genetic loss of CaaX protease function. Consistent with Rce1p being the *in vivo* target of the inhibitors, we observe that compound-induced delocalization is suppressed by increasing the gene dosage of *RCE1*. Moreover, we observe that Rce1p biochemical activity associated with inhibitor-treated cells is inversely correlated with compound dose. Genetic loss of CaaX proteolysis results in mistargeting of GFP–Ras2p to subcellular foci that are positive for the endoplasmic reticulum marker Sec63p. Pharmacological inhibition of CaaX protease activity also delocalizes GFP–Ras2p to foci, but these foci are not as strongly positive for Sec63p. Lastly, we demonstrate that heterologously expressed human Rce1p can mediate proper targeting of yeast Ras and that its activity can also be perturbed by some of the above inhibitors. Together, these results indicate that disrupting the proteolytic modification of Ras GTPases impacts their *in vivo* trafficking.

Keywords

CaaX protein; isoprenylation; post-translational modification; Ras; Rce1p

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Supporting Information

Supporting information may be found in the online version of this article.

Introduction

Members of the Ras GTPase family and certain other isoprenylated proteins are synthesized as precursors having a highly degenerate C-terminal tetrapeptide CaaX motif (C = cysteine; a aliphatic amino acid; X = one of several amino acids). These so-called CaaX proteins undergo an ordered series of modifications (Winter-Vann and Casey, 2005; Young *et al.*, 2001). The first of these modifications is the covalent attachment of an isoprenoid lipid to the sulphhydryl side-chain of the landmark cysteine of the CaaX motif. This event is mediated by either the farnesyltransferase (FTase; a heterodimer of Ram1p and Ram2p) or the geranyl-geranyl transferase I (GGTase I; a heterodimer of Ram2p and Cdc43p). Isoprenylation is followed by an endoproteolytic event that removes the last three residues of the motif (i.e. aaX). The Ras-converting enzyme (Rce1p) and sterile mutant 24 (Ste24p) can independently mediate this cleavage, despite being otherwise unrelated enzymes (Boyartchuk *et al.*, 1997; Tam *et al.*, 1998). Rce1p and Ste24p are only partly redundant. While both enzymes can promote production of the yeast **a**-factor mating pheromone, each also has specific target substrates (Tam *et al.*, 2001). For example, Rce1p cleaves Ras precursors (Otto *et al.*, 1999; Schmidt *et al.*, 1998), while mammalian Ste24p (ZmpSte24) is involved in maturation of mammalian lamin A (Corrigan *et al.*, 2005; Young *et al.*, 2006) and is genetically implicated in yeast membrane protein topogenesis (Tipper and Harley, 2002). Completing the set of modifications is carboxyl methyl esterification of the isoprenylated cysteine by the isoprenyl cysteine methyltransferase (ICMT; Ste14p).

The isoprenoid transferases are cytosolic enzymes, while the CaaX proteases and ICMT are integral membrane proteins that localize to the endoplasmic reticulum (ER) (Bracha-Drori *et al.*, 2008; Dai *et al.*, 1998; Romano *et al.*, 1998; Schmidt *et al.*, 1998). These observations and the fact that the Ras GTPases are predominantly plasma membrane-localized imply that CaaX proteins must be trafficked to their final destinations (Schmidt *et al.*, 1998). The nature of this trafficking is largely uncharacterized for most CaaX proteins. Where this issue has been investigated, Ras has typically been the enzyme of focus. In mammalian systems, N-Ras and H-Ras, but not K-Ras4B, rely on the secretory pathway for targeting to the plasma membrane (Choy *et al.*, 1999). However, the plasma membrane is not the only site capable of anchoring Ras signalling platforms. In yeast, ample evidence exists for the localization of Ras to the ER and mitochondria, but the manner by which Ras is trafficked to these destinations has not yet been elucidated (Onken *et al.*, 2006; Sobering *et al.*, 2003). These atypically localized Ras signalling platforms can elicit cellular responses distinct from that of plasma membrane-based platforms (Philips, 2005).

Disrupting the post-translational modification of CaaX proteins often yields intermediates having disrupted function (Boyartchuk *et al.*, 1997; Caplan *et al.*, 1992b; Kato *et al.*, 1992; Wright and Philips, 2006). Mislocalization of CaaX protein intermediates may in part explain observed protein dysfunction (Bergo *et al.*, 2000; Boyartchuk *et al.*, 1997; Winter-Vann *et al.*, 2005). Because of the prominent and active role of CaaX proteins in cellular transformation (i.e. the Ras GTPases), disrupting the post-translational modification of CaaX proteins is perceived as an anti-cancer strategy (Winter-Vann and Casey, 2005). To date, most drug discovery efforts have centred on FTase inhibitors (FTIs) because the FTase is the best-characterized enzyme of those that modify CaaX proteins (Cox and Der, 2002;

Zhu *et al.*, 2003). Nonetheless, inhibitors of the CaaX proteases and ICMT are being developed that likely hold equal promise (Anderson *et al.*, 2005; Dolence *et al.*, 2000; Manandhar *et al.*, 2007; Porter *et al.*, 2007; Schlitzer *et al.*, 2001; Winter-Vann *et al.*, 2005).

We previously screened a small-compound library for *in vitro* inhibitors of yeast Rce1p (Manandhar *et al.*, 2007). We identified nine compounds (compounds **1–9**) with *in vitro* IC_{50} values in the low micromolar range. In the present study, our goal was to determine whether these nine inhibitors can be used to modulate the *in vivo* activity of Rce1p. Through GFP–Ras reporters and yeast of various deletion backgrounds, we demonstrate that the extent of post-translational modification can significantly impact the intracellular targeting of CaaX proteins and that these effects can be pharmacologically phenocopied. Our results support the hypothesis that small-molecule CaaX protease inhibitors can be used to alter CaaX protein localization and suggest that Rce1p inhibitors have potential as chemotherapeutics.

Materials and methods

Yeast strains and media

The yeast strains used in this study are listed in Table 1. Plasmid-bearing versions of these strains were generated according to published methods (Elble, 1992). Strains were routinely grown at 30°C, with the exception of DF1 and plasmid transformants of this strain that were grown at 25°C. Yeast were cultured using YEPD or synthetic complete drop-out glucose-based medium (e.g. SC–Ura and SC–Leu), as previously described (Michaelis and Herskowitz, 1988). Experiments involving galactose-induced protein expression used synthetic media lacking glucose and uracil (or leucine) but containing 2% galactose, 1% glycerol and 1% ethanol (i.e. SGal–Ura and SGal–Leu).

Plasmids

The plasmids used in this study are listed in Table 2. pWS750 was constructed by recombination-mediated plasmid construction. In brief, the *Bgl*II fragment encoding P_{GAL}–*GFP–RAS2* from pWS270 was co-transformed into yeast with *Not*I-and *Xho*I-gapped pRS315. The *Bgl*II fragment contains significant sequence homology at either end to sequences of pRS315, which facilitates recombination events *in vivo*. Recombinant plasmid clones were recovered from yeast surviving SC–Leu selection, amplified in *E. coli* DH5*α*, and correct clones identified by restriction digest analysis. pWS751 was constructed by replacing the *RAS2* open reading frame (ORF) in pWS750 with that of *YCK2* by subcloning. A PCR fragment encoding *YCK2* (amplified from pJB1) contained an *Xba*I site immediately preceding the start codon and a *Not*I site immediately following the stop codon of the *YCK2* ORF; these sites flank the *RAS2* ORF in pWS750. pWS808 was created by recombination-mediated plasmid construction, such that the *RAS1* ORF replaced the *RAS2* ORF in pWS270 (Oldenburg *et al.*, 1997). The *RAS1* ORF was amplified by PCR (from YEp*RAS1*), such that the product contained flanking sequences that were homologous to sequences flanking the *RAS2* ORF in pWS270. Yeast were co-transformed with the appropriate PCR product and *Xba*I-linearized pWS270, plasmids were recovered from clones surviving SC–Ura selection and the plasmids were evaluated by restriction digest analysis. pWS881 was constructed in a similar manner to pWS750, except that pWS808 was

used to generate the insert. The regions encoding the CaaX motifs in the above plasmids were verified by DNA sequencing.

GFP–CaaX protein localization assay

An inducible GFP–Ras2p reporter (pWS270 or pWS750) was used to determine the localization of Ras2p in various genetic backgrounds and the ability of chemical agents to disrupt Ras2p localization in wild-type cells (Manandhar *et al.*, 2007). Reporters encoding GFP–Ras1p, GFP–Yck2p and Sec63p–GFP were evaluated in a similar manner. In brief, mid-log yeast cells containing the appropriate reporter were harvested, washed twice with sterile H_2O and incubated in SGal–Ura (or SGal–Leu) for approximately 6 h at 30°C to induce expression of the inducible GFP reporters. Approximately 60–80% of cells had strong GFP fluorescence with this protocol. Sec63p–GFP localization was examined under both glucose and galactose conditions (SC–Ura and SGal–Ura, respectively). The expression pattern of GFP reporters was visualized using a Zeiss Axioskop 2 Mot Plus microscope equipped with fluorescence optics. Images were captured at × 100 magnification (plan Apochromat objective, numerical aperture 1.4), using appropriate filters with an ORCA-AG digital camera (Hamamatsu, Japan) and IPLab Spectrum software. For each experiment, at least three cell fields were taken, from which representative images were selected. Images for co-localization studies were false-colourized as appropriate, using Adobe Photoshop CS2 v. 9.0. Merged images were created by overlapping the false-colourized GFP image (50% opacity) over that of the RFP image, using Adobe Photoshop CS2.

For instances where compounds were evaluated, cells were pretreated with chemical agents for 1 h at the appropriate induction temperature at their *in vivo* EC_5 (concentration where minimum growth inhibition is observed) or up to 50 μM in some instances. Following pretreatment, the cells were washed with sterile distilled H_2O and resuspended in appropriate SGal-media as described above. EC_5 concentrations were extrapolated from the EC_{50} dataset using the equation $EC_F = (EC_{50})[F(100 - F)^{-1}]^{1/H}$, where the percentage value desired (F) was set to 5 and the Hill coefficient (H) was assumed to be 1 (Manandhar *et al.*, 2007; Motulsky, 2005). As indicated in the appropriate figure legends, the pretreatment time or induction time varied. Where indicated, cells were treated before microscopic evaluation with 1 μg/ml propidium iodide (Sigma) for 10 min (Marobbio *et al.*, 2003; Zhang, 2006).

Toxicity studies

The effects of exposure to small-molecule CaaX protease inhibitors on cell growth and viability were determined using growth assays. To assess transient exposure, EG123 transformed with pWS270 was grown to mid-log phase (A_{600} = 0.5–1.0) in SC–Ura, the culture split into 0.1 ml aliquots and the aliquots treated for 1 h with a range of inhibitor concentrations (0–2 mM) in triplicate. Dilutions of the treated cultures were plated on YEPD to evaluate the number of viable cells, as determined by colony counting after 48 h growth at 30°C. Colony counts were graphed vs. compound concentration, using GraphPad Prism 4.0 (four-parameter logistic equation) to determine EC_{50} toxicity values. The EC_5 concentrations of compounds (calculated as described above) were re-evaluated by the plate viability protocol after 6 h of galactose induction (7 h total exposure to compound) to

validate the limited toxicity at these lower concentrations. The viabilities observed for EC_5 treatment conditions are reported as a percentage relative to a DSMO-treated control. To assess continuous exposure, a saturated yeast culture of EG123 transformed with pRS316 was highly diluted (1: 4000) into fresh SC–Ura and split into equal-volume aliquots (2 ml). The individual aliquots were cultured on a rolling drum mixer in the presence of compounds at the indicated concentrations until DMSO-treated cultures reached the density range A_{600} = 0.75–1.0 (approximately 22 h). A graph of A_{600} vs. concentration of inhibitors was plotted, and EC_{50} toxicity values of the inhibitors were determined using GraphPad Prism 4.0, as described above.

Confocal laser scanning microscopy

For experiments involving confocal microscopy, yeast were cultured and manipulated as described above before being imaged, using a Zeiss LSM510 META confocal microscope with a × 100 α-Plan Fluar 1.45 NA oil objective lens at a resolution of 0.04 μm per pixel. GFP was excited with a 488 nm laser (0.3–4% intensity) and light emission captured through a 505–530 nm BP filter. The laser intensity chosen yielded a linear fluorescence response by the reporter (see Supporting information, Figure S1). The relative distribution of GFP–Ras2p was determined with Zeiss LSM imaging software from a minimum of five images. The values obtained were used to calculate the percentage of total fluorescence associated with the plasma membrane, where delocalized fluorescence was defined as any fluorescence 0.75 μm or more from the peak boundaries. The error in the associated bar graphs represents the standard error of the mean (SEM) for each value.

Biochemical assessment of Rce1p activity in yeast treated with CaaX protease inhibitors

A highly diluted culture of yeast over-expressing Rce1p-HA (SM3614 transformed with pWS479) was grown for approximately 16 h at 30°C to mid-log (A_{600} = ~1.0) in the presence of DMSO or compounds **4** or **8** (at their EC_{10} and EC_{50} values, which are 6.8 and 61 μM for compound **4** and 10.8 and 97 μM for compound **8**, respectively). The cells were harvested by centrifugation (3000 × *g* for 5 min), washed once with distilled water and frozen at −80°C. Thawed cells were used to prepare an ER-enriched membrane fraction, as previously described (Manandhar *et al.*, 2007; Porter *et al.*, 2007). In brief, the cells were converted to spheroplasts with Zymolyase 100T (Cape Cod Inc., East Falmouth, MA, USA), lysed by mechanical agitation with silica beads (i.e. bead beating), and debris and unbroken cells removed by low-speed centrifugation (500 × *g* for 10 min). Membranes were recovered from the lysate by centrifugation (16 000 × *g* for 15 min), washed with lysis buffer (50 mM Tris, pH 7.5, 0.2 M sorbitol, 1 mM EDTA, 0.02% NaN_3) supplemented with protease inhibitors (1 mM PMSF and 1 μg/ml each of chymostatin, leupeptin, pepstatin and aprotinin), re-isolated by centrifugation and resuspended to 1 mg/ml in lysis buffer.

To assess the biochemical effects of compounds, Rce1p activity was analysed in duplicate, using a fluorescence-based assay using equivalent amounts of membrane material (0.25 mg/ml) isolated as described above from each treatment condition (Manandhar *et al.*, 2007). The amount of activity observed was further normalized to the amount of Rce1p present in each sample. Rce1p amounts were quantified using ImageJ (National Institutes of Health,

Bethesda, MD, USA) from digital images of Rce1p immunoblots (Molecular Image FX imaging system, Bio-Rad).

Results

GFP–Ras2p localization varies with its degree of post-translational modification

To qualitatively and quantitatively investigate the impact of post-translational modification on Ras subcellular localization in yeast, we expressed GFP–Ras1p and GFP–Ras2p in yeast having specific defects for each of the three steps associated with CaaX protein modification. In yeast, as in other eukaryotic organisms, the Ras GTPases predominantly decorate the cytosolic face of the plasma membrane (Figure 1A, B, WT). Quantification of GFP–Ras2p fluorescence reveals that most (94%) of the total fluorescence in a wild-type background is detected within 0.75 μm of the plasma membrane, as defined by readily identifiable peak boundaries in cross-sectional fluorescence profiles of individual cells; this type of analysis is the first quantitative measure of yeast Ras localization of which we are aware. In an FTase-deficient strain (*ram1*Δ), a strong cytosolic localization pattern was observed for the Ras reporters, and quantification of the GFP–Ras2p signal revealed that only 19% of total fluorescence was near the plasma membrane. The pattern is consistent with the role of isoprenoid lipids in mediating the membrane association of Ras proteins. Importantly, the genetic background deficient for protein isoprenylation did not, in and of itself, cause an alteration in plasma membrane composition that precluded affinity for lipid-modified proteins, as revealed by the normal localization pattern of GFP–Yck2p. Yck2p is a palmitoylated and plasma membrane-localized protein whose maturation does not depend upon the enzymes required for the modification of Ras and other CaaX proteins (Babu *et al.*, 2004).

In the absence of CaaX protease activity, anywhere from one to several subcellular punctate structures were observed for both of the Ras GTPases (*rce1*Δ *ste24*Δ; Figure 1A, B). Quantification of GFP–Ras2p subcellular distribution revealed that <23% of the associated fluorescence was near the plasma membrane. By comparison, GFP–Yck2p localization appeared qualitatively normal. For GFP–Ras2p, the observed punctate pattern can be contrasted to a more diffuse delocalized pattern observed in a CaaX protease-deficient yeast strain of different lineage (Boyartchuk *et al.*, 1997). To resolve the individual impact of each CaaX protease, GFP–Ras1p and GFP–Ras2p were expressed in yeast lacking either Rce1p (*rce1*Δ) or Ste24p (*ste24*Δ). Ste24p is not required for the proteolytic maturation of Ras (Boyartchuk *et al.*, 1997; Schmidt *et al.*, 1998). As expected in the absence of Ste24p, GFP–Ras localized predominantly to the plasma membrane, as determined by both qualitative and quantitative measures (88% near plasma membrane for GFP–Ras2p). In the absence of Rce1p, GFP–Ras was clearly delocalized (29% near plasma membrane for GFP–Ras2p), but the pattern was more diffuse than seen in the absence of both CaaX proteases and was not generally associated with punctate foci. The explanation for the differential pattern in the *rce1Δ* by comparison to a complete CaaX protease-deficient background is unknown, but the result suggests that Ste24p may play an indirect role in the localization of Ras2p intermediates. Complementing these observations, we noted that the GFP–Ras2p pattern reverted to the *ste24Δ* pattern (i.e. plasma membrane-associated) when yeast Rce1p was co-

expressed with the reporter in the CaaX protease-deficient background (*rce1*Δ *ste24*Δ; Figure 1C); co-expression of yeast Ste24p led to a pattern similar to that seen in the *rce1Δ* background.

In cells lacking ICMT activity, the Ras GTPases were also delocalized (*ste14-3*; Figure 1A, B). The diffuse distribution pattern was qualitatively similar to that observed for *rce1Δ*. Quantification of GFP–Ras2p subcellular distribution in the ICMT-deficient background revealed that 29% of the associated fluorescence was near the plasma membrane. Other CaaX proteins are known to depend on ICMT for proper localization. For example, the isoprenylated plant calmodulin CaM53 is targeted to the plasma membrane when heterologously expressed in yeast, but is mistargeted to the endomembranes in the absence of ICMT activity (Rodriguez-Concepcion *et al.*, 2000). Mammalian Ras isoforms are similarly mislocalized in the absence of ICMT activity (Bergo *et al.*, 2000; Winter-Vann *et al.*, 2005).

Together, our results indicate that isoprenylation, CaaX proteolysis and ICMT activity are critical for the proper plasma membrane localization of Ras1p and Ras2p but not the palmitoylated protein Yck2p. Our qualitative observations indicate that defects in each of these activities impart distinct biophysical properties to the Ras intermediate that are reflected in distinct subcellular localization patterning. Our quantitative observations indicate that each activity has approximately equal weight in helping promote the plasma membrane localization. This latter observation suggests that inhibitors of the various steps of the CaaX protein modification pathway are likely to have synergistic effects.

Certain CaaX protease inhibitors disrupt GFP–Ras2p localization

We previously identified nine compounds from the NCI Diversity Set Library that inhibit the *in vitro* activity of Rce1p (Manandhar *et al.*, 2007). In some instances, these compounds also inhibited to varying degrees the *in vitro* activities of the Ste24p CaaX protease (compounds **3–9**) and Ste14p ICMT (compounds **3–5, 8** and **9**). Compounds **3–5, 8** and **9** also inhibited the protease activity of trypsin. To investigate the *in vivo* properties of these compounds, we examined whether any of the nine compounds were cytotoxic and subsequently whether any could disrupt Ras2p localization inside yeast cells.

The toxicity of these agents toward yeast was examined for both transient (1 hr) and continuous exposures (Table 3). Transient exposures revealed that compounds **1, 2** and **5** were non-toxic up to 500 μM, the highest concentration evaluated. Compounds **3, 4** and **6–9** had varied toxicity, with compound **6** being the most toxic. A toxicity profile was also obtained for continuous exposure. This analysis also revealed dose-dependent toxic effects for many of the compounds. Compounds **6, 7** and **9** were most toxic under conditions of continuous exposure. Compounds **1** and **2** had no detectable toxicity. Generally, compounds with relatively lower EC_{50} values in the transient exposure study had relatively lower EC_{50} values in the continuous exposure study. Compound **7** was a notable exception, but it could not be reliably evaluated as it did not yield a sigmoidal response in transient exposure toxicity studies. The toxicity profiles and the reported observation that Rce1p is a non-essential protein suggest that some of the evaluated compounds (e.g. **6, 7** and **9**) have off-targets that include essential processes. Interestingly, the tolerance of yeast to a particular

compound did not correlate with observed *in vitro* specificity against CaaX-modifying enzymes (Manandhar *et al.*, 2007). For example, the somewhat selective *in vitro* Rce1p inhibitor represented by compound **6** was cytotoxic, whereas the relatively non-specific compound **5** was one of the least toxic compounds.

We next investigated the ability of compounds to disrupt yeast Ras2p localization. In order to minimize artifacts associated with cytotoxicity, we used concentrations where 95% or greater viability was expected after transient exposure to a compound. At these minimally toxic (EC_5) concentrations, compounds **4** and **6–9** exerted GFP–Ras2p delocalization effects consistent with loss of CaaX protease activity (i.e. punctate patterning), while others were seemingly ineffective (Figure 2A, Table 3). Delocalization was observed despite the lack of continuous exposure to compounds, suggesting that these compounds are cell-permeable and tightly or otherwise irreversibly bind to their targets. None of the compounds induced the diffuse GFP–Ras2p localization pattern observed in *rce1Δ* cells. We attribute this to the lack of specificity of these compounds. Those that induced delocalization of GFP–Ras2p inhibit both Rce1p and Ste24p *in vitro* (Manandhar *et al.*, 2007). Thus, they are expected to pharmacologically induce the pattern observed in a CaaX protease-deficient background (*rce1*Δ *ste24*Δ). None of the compounds interfered with GFP–Yck2p localization, suggesting that the compounds are not generally disrupting plasma membrane architecture or dynamics.

To assess whether compounds **1, 2, 3** and **5** had any capability of inducing GFP–Ras2p delocalization, we varied our protocol for compound concentration and exposure times, and evaluated the effect of additives that aid in dispersal of compounds in solution. For example, we examined the effect of continuous rather than transient exposure of cells to compounds, using doses as high as 50 μM (**1, 2** and **5**) or 25 μM (**3**; higher doses were toxic). These changes had no observable impact on GFP–Ras2p delocalization. Likewise, increasing the transient dose (up to 100 μM) and duration (3 h) of compound treatment, alone or in combination, also failed to induce an effect (data not shown). The presence of a non-toxic amount of SDS (0.003%), however, did yield a delocalization phenotype in conjunction with compound **3** at a concentration of 25 μM (Figure 2B). No such effect was observed with compounds **1, 2** and **5**, even when evaluated in the presence of other detergents (e.g. NP-40, Tween 20, TX-100 or deoxycholate). Based on our combined observations, we conclude that compounds **4** and **6–9** are highly cell-permeable, compound **3** is permeable when applied with a dispersal agent, and compounds **1, 2** and **5** are either impermeable, unstable or otherwise ineffective at interfering with proteolysis of the Ras2p CaaX protein *in vivo*.

Compounds **4** and **8** were evaluated in more detail because they were among the least toxic compounds that could induce a Ras delocalization phenotype. Treatment of wild-type cells with these compounds induced quantifiable delocalization effects comparable to that observed in the CaaX protease-deficient background (Figure 2C). By comparison with DMSO-treated cells, where 93% of GFP–Ras2p was localized near the plasma membrane, the reporter was localized 44% and 34% upon treatment with compounds **4** and **8**, respectively. Neither compound induced a delocalization phenotype to the extent observed with complete loss of CaaX proteolytic activity (*rce1*Δ *ste24*Δ; 22% near the plasma membrane).

To support our contention that compounds are targeting Rce1p intracellularly, we predicted that increasing the dosage of Rce1p would render inhibitors less effective. We tested this prediction using compound **4** and plasmid-based expression of Rce1p, which increases Rce1p production substantially over that observed when only the genomic copy of *RCE1* is available (Schmidt *et al.*, 1998). With this approach, we observed that compound-induced Ras2p delocalization in wild-type control cells (*CEN*) could be alleviated under conditions of increased *RCE1* gene dosage (*CEN RCE1*), suggesting that observed inhibitor effects are indeed Rce1p-dependent (Figure 2D). The most parsimonious explanation for our observations is that the Ras delocalizing agents described here are interfering with Rce1-dependent CaaX proteolysis.

GFP–Ras2p delocalization is dependent on the duration of compound exposure

In order to assess how quickly certain compounds could induce a Ras delocalization phenotype, we carried out time-course experiments with compounds **4** and **8**. Similar time-dependent effects were seen with both compounds. As expected, extremely short pretreatment times (1 min) did not delocalize the reporter from the plasma membrane (Figure 3A). Punctate-patterning effects were observed with pretreatments as short as 20 and 40 min with compounds **4** and **8**, respectively. The punctate patterns appeared without the prior appearance of the diffuse intracellular pattern observed in *rce1Δ* cells. As discussed above, we believe this to be due to the fact that compounds **4** and **8** inhibit both Rce1p and Ste24p, in effect pharmacologically phenocopying a CaaX protease-deficient background. We also determined that compounds **4** and **8** could not dislodge GFP–Ras2p, which had been previously targeted to the plasma membrane, as judged by experiments in which chemical agents were added to wild-type cells after induction of GFP–Ras2p expression (Manandhar and Schmidt, unpublished observation).

Additional time-course studies indicated that the compound-induced punctate patterning for GFP–Ras2p was temporal. After prolonged (9 h) induction in galactose-medium or a recovery in glucose-containing medium (3 h) after a standard 6 h induction, the plasma membrane localization pattern was predominant in the population (Figure 3B). Several possibilities could explain this observation. One is that there is synthesis of new, and thus uninhibited, molecules of Rce1p during the induction period, which are free to act upon the GFP–Ras2p intermediate accumulated at punctate sites. Alternatively, there could be chemical breakdown or metabolism of the compound. Regardless of the reason for the temporal effect, our observations suggest that punctate structures are dynamic in nature, which we infer can only occur in live cells. To further confirm that toxicity was not a contributing factor to our observations, we incubated compound treated yeast with the nucleic acid dye propidium iodide, which stains dead yeast. Cells with GFP–Ras2p punctate patterning went unstained, further supporting a lack of toxicity under the conditions tested, whereas dead cells displaying whole cell patterning were stained by the dye (Figure 3C).

Yeast Rce1p activity is reduced in compound treated cells

The ability of certain Rce1p inhibitors to delocalize GFP–Ras2p *in vivo*, and the observation that these effects are time-dependent, suggest that these particular compounds are cell-permeable. To directly assess whether intact yeast cells had reduced Rce1p expression

and/or activity after exposure to these compounds, the Rce1p-enriched membrane fraction was isolated from cells treated with or without these agents. For the purposes of this experiment, Rce1p was overexpressed in the cells, which was necessary for measuring Rce1p activity using our fluorescence-based biochemical assay. Membranes derived from cells that were treated continuously with compounds **4** and **8** had the same level of Rce1p expression as control membranes (Figure 4A) but had significantly reduced *in vitro* activity, proportional to the amount of inhibitor used to treat the cells (Figure 4B). Interestingly, membranes derived from cells treated with compound **8** had a readily observable yellow-orange tint consistent with the colour of this compound; no such colour was associated with membranes derived from cells treated with colourless compound **4** (Manandhar and Schmidt, unpublished observation). Together, our observations are consistent with compounds **4** and **8** being cell-permeable, and suggest that they inhibit the *in vivo* activity of Rce1p by either directly modifying Rce1p or indirectly disrupting local membrane architecture that is essential for Rce1p function.

Human Rce1p can promote the plasma membrane localization of GFP–Ras2p

Several observations suggest that heterologously expressed human Rce1p can substitute for yeast Rce1p in Ras2p maturation. First, H-Ras can substitute for the activity of yeast Ras2p, implying that H-Ras is likely recognized by the native CaaX modification enzymes of yeast (Kataoka *et al.*, 1985). Second, human Rce1p is functional in yeast, as judged by its ability to effectively process the farnesylated **a**-factor mating pheromone (Plummer *et al.*, 2006). Following up on these observations, we observe that human Rce1p can promote proper plasma membrane localization of GFP–Ras2p in yeast devoid of the yeast CaaX proteases (Figure 5A, DMSO-treated condition). We previously observed that, similar to yeast Rce1p, the *in vitro* activity of human Rce1p is strongly inhibited by compounds **1–9** (Manandhar *et al.*, 2007). Therefore, we predicted that the GFP–Ras2p reporter could be used to monitor the inhibitor sensitivity of human Rce1p *in vivo*. Indeed, compounds **3, 4, 6** and **9** induced GFP–Ras2p delocalization in cells that heterologously express human Rce1p (Figure 5A). Compound **3** induced delocalization only when SDS was present (Figure 5B). Compounds **7** and **8** were ineffective against human Rce1p when used at EC_5 concentrations (11.1 and 5.1 μM, respectively) that induced a uniform delocalization phenotype in the context of yeast Rce1p. However, a higher dose (25 μM) of compound **7** resulted in approximately 25% of cells having a diffuse and occasional punctate GFP–Ras2p patterning (Figure 5B). Treatment with higher doses was toxic to cells. Similar observations were made for compound **8**. The disparity between human and yeast Rce1p in response to compounds **7** and **8** may reflect unequal expression levels of the enzymes *in vivo*. Yeast Rce1p is expressed from a single genomic copy, whereas human Rce1p is expressed from a multi-copy plasmid and a strong promoter. Target specificity differences are also possible. In fact, we have observed, both *in vitro* and in a cell-based assay, that trypanosomal Rce1p is more sensitive to compound **7** as compared to human Rce1p (Mokry *et al.*, 2009). Together, our observations indicate that GFP–Ras2p can be a useful reporter for human Rce1p activity and that the subcellular distribution of GFP–Ras2p can be used as an indicator for determining the potency of compounds in disrupting human Rce1p activity.

Ras2p and the ER-specific marker Sec63p co-localize to subcellular foci in the absence of CaaX proteolysis

The fact that the yeast CaaX proteases are ER-localized is suggestive that non-proteolysed CaaX proteins might be way-stationed at the ER. The localization pattern of GFP–Ras2p in a CaaX protease-deficient background (*rce1*Δ *ste24*Δ), however, is not typical of ER-localized proteins, such as Sec63p, Kar2p, Rce1p, Ste24p and Ste14p (Prinz *et al.*, 2000; Romano *et al.*, 1998; Schmidt *et al.*, 1998). To assess the pattern of the ER in our strain background and under our assay conditions, we evaluated Sec63p–GFP, which is routinely used as a marker protein for the yeast ER (Prinz *et al.*, 2000). A typical ring-like ER localization pattern was observed for the constitutively expressed reporter in genetic backgrounds defective for the various steps associated with CaaX protein modification and in compound treated wild-type cells (Figure 6A). The CaaX protease-deficient background additionally displays occasional foci similar in appearance to those observed for GFP–Ras2p in this background. Interestingly, foci became the predominant Sec63p-positive structure when CaaX protease-deficient yeast were subjected to the galactose-induction protocol typically used in conjunction with our GFP–Ras2p localization studies, suggesting an unusual ER architecture under these conditions (Figure 6B).

The Sec63p–GFP foci apparent in CaaX protease-deficient yeast appeared similar enough to those observed for GFP–Ras2p to suggest that they originated from the same source. We investigated this issue through co-localization studies of GFP–Ras2p and Sec63p–RFP, using CaaX protease-deficient yeast (*rce1*Δ *ste24*Δ) and wild-type yeast that had been treated with and without compounds **4** and **8** (Figure 6C). In CaaX protease-deficient yeast, a significant proportion of cells (63%, $n = 51$) had signal overlap between Sec63p–RFP and GFP–Ras2p. In compound treated wild-type cells, GFP–Ras2p and Sec63-RFP localized to distinct non-overlapping foci (compound **4**, 9% overlap, $n = 103$; compound **8**, 13% overlap, $n = 32$). Therefore, it appears that GFP–Ras2p delocalizes predominantly to ER-derived structures in the complete absence of the CaaX protease activity and to an undefined site in compound treated cells. The lack of signal overlap between GFP–Ras2p and Sec63p–RFP upon compound treatment may reflect incomplete inactivation of one or both of the CaaX proteases *in vivo*, the temporal nature of delocalization, and/or the net effect of these compounds on other targets, such as Ste14p, within the cell.

Discussion

The most important observations of this study pertain to the impact of CaaX proteolysis on Ras localization. To our knowledge, we have provided the first quantification of Ras delocalization in the absence of the three steps in CaaX protein processing: isoprenylation, proteolysis and carboxylmethylation. Our results demonstrate that Ras2p is significantly delocalized from the plasma membrane in the absence of any of the three steps, despite the observation that the subcellular distribution of our GTPase reporters varied somewhat depending on the defective step. Together, these findings suggest that inhibitors of specific steps associated with CaaX protein processing are likely to have synergistic effects when combined.

We have also demonstrated that small-molecule agents can chemically induce defects in CaaX proteolysis that, at first glance, appear to pharmacologically phenocopy the Ras2p delocalization effects seen in CaaX protease-deficient yeast. The most specific of the Rce1p inhibitors, compounds **1** and **2**, did not elicit a change in Ras2p localization despite numerous attempts to improve the solubility and uptake of these compounds by yeast cells. We previously reported that compounds **3–5, 8** and **9** have broad *in vitro* specificity, including the ability to inhibit Ste24p and Ste14p (Manandhar *et al.*, 2007). Several compounds have also been identified as inhibitors of other enzymes, e.g. compounds **4** and **5** inhibit DNA topoisomerases (Bond *et al.*, 2006; Stivers and Kwon, 2006). Nevertheless, the compound induced Ras2p delocalization observed *in vivo* appears Rce1p-dependent, as inferred from gene dosage experiments, and not the indirect result of disrupting the plasma membrane architecture, as inferred from the normal subcellular distribution of Yck2p. The *in vivo* targeting of Rce1p by these agents is further supported by the reduced biochemical activity of Rce1p observed in membranes derived from compound treated yeast. Combined, our observations and the fact that the activity of human Rce1p can be monitored using the yeast GFP–Ras2p system suggest the possibility of using automated microscopy methods for identifying cell-permeable human Rce1p inhibitors as part of drug discovery studies. As for our current set of compounds, their ability to induce robust delocalization at low micromolar concentrations is intriguing, but the associated toxicity of most clearly tempers the potential of these agents as lead compounds in drug development trials. Whether these compounds induce delocalization effects in, and are toxic to, mammalian cells remains to be determined.

An intriguing observation made during the course of this study is that the classic ER marker Sec63p partly localizes to subcellular foci in the absence of CaaX proteolytic activity under standard culture conditions (i.e. glucose-containing media) (Figure 6A; *rce1Δ ste24Δ*). These foci coexist with a more traditional ring-like ER pattern. A change in carbon source, however, results in the foci becoming the prominent Sec63p-positive feature in the absence of CaaX proteolytic activity and a transient feature in wild-type cells (Figure 6B). The foci are not present in *rce1Δ* or *ste24Δ* single mutants grown in glucose (this study) or galactose (Manandhar and Schmidt, unpublished observation). The ER is known to readily adapt its structure to changing cellular needs (Federovitch *et al.*, 2005). We propose a model in which the classic ER pattern is dynamically remodelled in response to a nutritional change (i.e. the replacement of glucose by galactose). This remodelling is short-circuited in the absence of CaaX protease activity, resulting in foci. GFP–Ras2p is seen to co-localize to these ER-foci in the absence of CaaX-proteolysis, where it is presumably anchored by the farnesyl moiety. Despite co-localization between Sec63p and Ras2p in a CaaX protease-deficient background, chemically induced Ras-containing foci do not contain significant amounts of Sec63p, and thus may be derived from a separate ER subdomain or other organelle. The reason for this discrepancy is unresolved, but could reflect redundancy in the ER remodelling system, incomplete pharmacological inactivation of CaaX proteolytic activity, or compounds acting promiscuously on other proteins (e.g. Ste14p) involved in Ras2p or Sec63p trafficking (Dong *et al.*, 2003; Wang and Deschenes, 2006). Further investigations are needed to understand this phenomenon in the context of distinct nutritional stresses and gene requirements.

As mentioned above, we have occasionally observed Sec63p-positive foci in the wild-type background upon a change in carbon source, but they are transient and relatively rare. The presence of foci thus seems to be a normal part of ER remodelling. Since foci are not persistent unless both CaaX proteases are absent, it can be reasoned that there may be CaaX proteins involved in this remodelling event that either directly participate or regulate other proteins in this process. A prime candidate is the chaperone Ydj1p, which is in part found at the ER membrane, where it is involved in ER preprotein translocation and the turnover of proteins, including those subject to ERAD (Caplan *et al.*, 1992a; Mandal *et al.*, 2008; Trueblood *et al.*, 2000; Youker *et al.*, 2004). Defective post-translation processing of Ydj1p, specifically lack of isoprenylation, disrupts the activation of certain Ydj1p-dependent proteins, so it is possible that a lack of CaaX proteolysis has a similar effect (Flom *et al.*, 2008). We do not know, however, which if any Ydj1p client may be specifically involved in ER remodelling. Unfortunately, the target specificities of the CaaX proteases have not been fully resolved in any system, which complicates the identification of candidate CaaX proteins involved in this proposed ER remodelling process. We envision several approaches that may lead to the identification of CaaX proteins involved in ER remodelling (e.g. high copy suppression studies) that could be utilized in future investigations of this phenomenon.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Dr M. P. Terns (University of Georgia) for access to a Zeiss Axioskop 2 Mot Plus microscope, Dr L. C. Robinson (LSU Health Sciences Center-Shreveport), Dr P. A. Silver (Harvard Medical School), Dr S. Yoshida (Pellman Laboratory, Dana-Farber Cancer Institute) and Dr S. Michaelis (Johns Hopkins University School of Medicine) for plasmids pJB1, pJK59 and pSM1960, respectively, and Dr R. Jensen (Johns Hopkins University School of Medicine) and members of the Schmidt laboratory (University of Georgia) for critical discussions and technical assistance. We also thank the NCI Developmental Therapeutics Program for access to chemical compounds. This work was supported by R01 and R03 grants (GM67092 and NS053625) from the National Institutes of Health (to W.K.S.).

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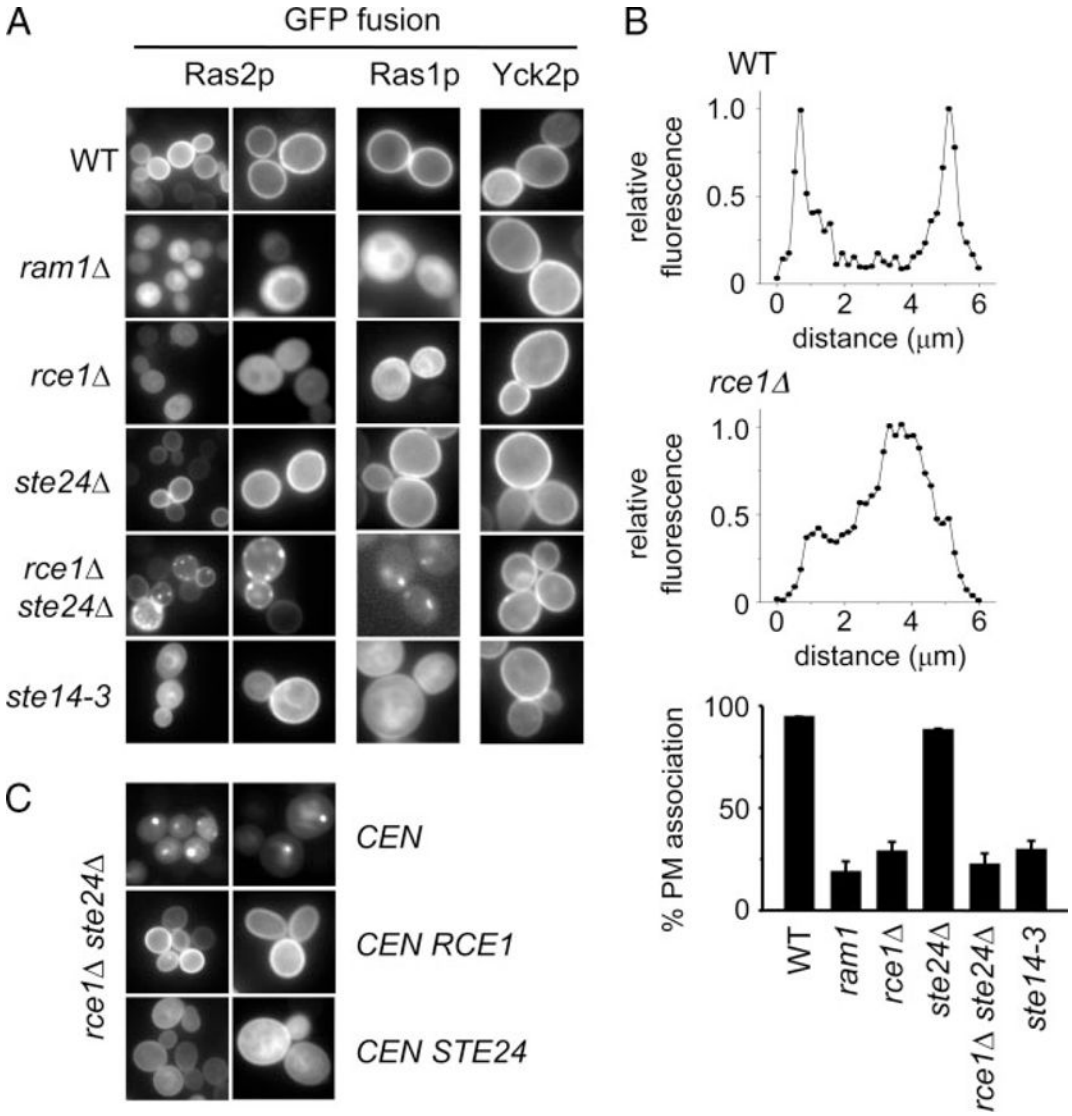

Figure 1.

The localization of GFP–Ras2p and GFP–Ras1p depends on proper post-translational modification. (A) GFP–Ras2p, GFP–Ras1p and GFP–Yck2p reporters were inducibly expressed in yeast that were wild-type (WT) or defective for the activity of the FTase (*ram1*Δ), one or both of the CaaX proteases (*rce1Δ, ste24Δ*) or the ICMT (*ste14-3*). The expression of the reporters, which were encoded behind the *GAL1* promoter, was induced in mid-log yeast by switching from glucose-to galactose-containing media. A field of cells and an enlarged image are shown for GFP–Ras2p expressed in each background. An enlarged image alone is shown for GFP–Ras1p and GFP–Yck2p that is representative of the field in the indicated background. Plasmid-transformed strains of EG123 (WT), DF1 (*ram1Δ*), SM1188 (*ste14-3*), SM3103 (*ste24*Δ), SM3613 (*rce1*Δ), and SM3614 (*rce1*Δ *ste24*Δ) were used. With the exception of DF1, the plasmids used were pWS270 (*GFP–RAS2*), pWS808 (*GFP–RAS1*) and pJB1 (*GFP–YCK2*). For DF1, the plasmids used were pWS750 (*GFP–RAS2*), pWS881 (*GFP–RAS1*) and pWS751 (*GFP–YCK2*). (B) Quantification of GFP–Ras2p delocalization. Confocal laser scanning microscopy was used to determine cross-

sectional fluorescence profiles of WT and *rce1*Δ yeast (top and middle panels, respectively) induced to express GFP–Ras2p. Data were collected using 1% of maximum laser strength at 488 nm. These profiles were used to calculate the amount of plasma membrane (PM)-localized fluorescence, which is graphed along with the standard error of the mean (bottom panel). The PM-localized fluorescence was defined as any fluorescence within 0.75 μm of the peak boundaries. The strains used were the same as those described in (A). (C) Add-back experiments confirm the dependence of GFP–Ras2p plasma membrane localization on Rce1p. A CaaX protease-deficient strain (*rce1*Δ *ste24*Δ; yWS164) was assessed for GFP–Ras2p localization as described in (A) after co-transformation with plasmids encoding Rce1p or Ste24p. The plasmids used were pRS416 (*CEN*), pSM1107 (*CEN STE24*), pSM1314 (*CEN RCE1*) and pWS750 (*GFP–RAS2*)

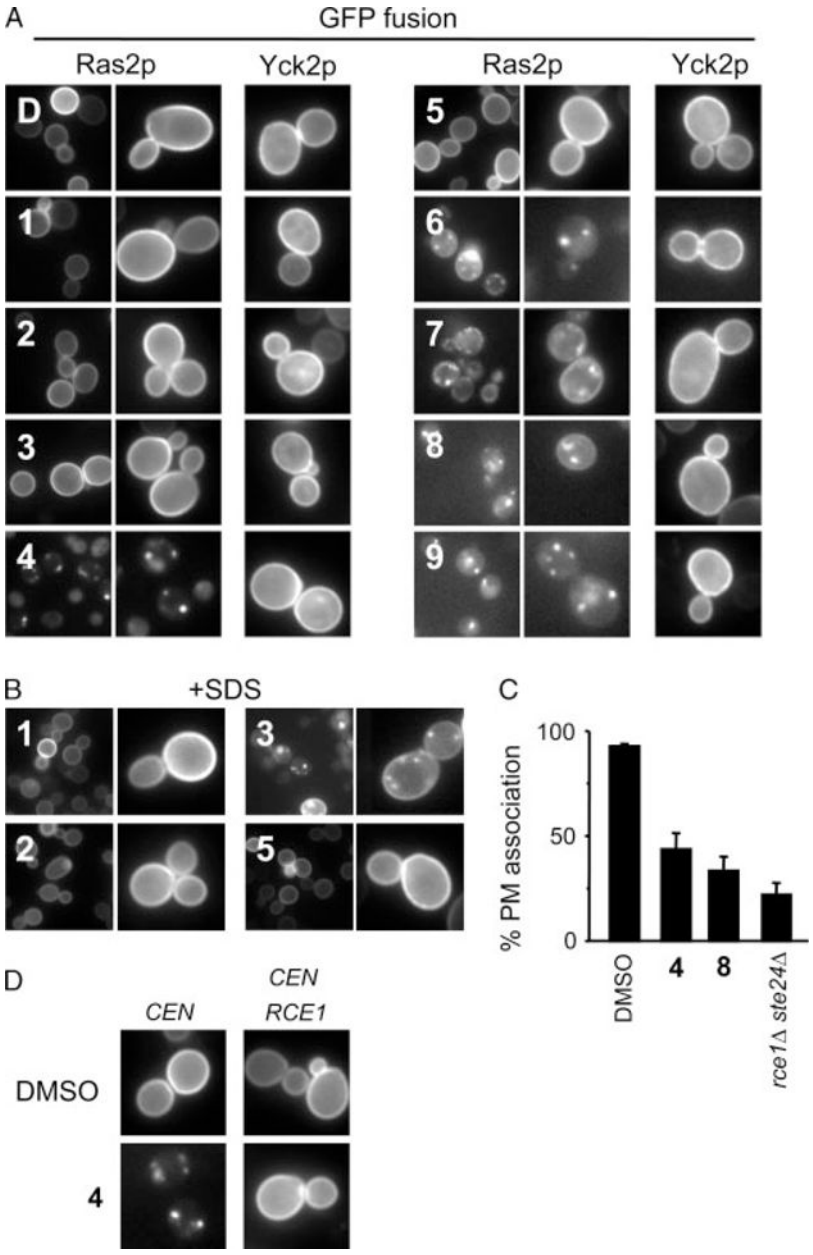

Figure 2.

GFP–Ras2p can be delocalized by chemical agents. GFP–Ras2p and GFP–Yck2p were inducibly expressed in wild-type yeast, as described in Figure 1, after a transient 1 h pretreatment with the indicated compound. The compounds were used at EC_5 concentrations, as calculated from transient exposure toxicity studies (see Table 3). (B) Compound **3** has latent GFP–Ras2p delocalization activity. GFP–Ras2p was inducibly expressed in wild-type yeast after 1 h pretreatment with 25 μM of the indicated compounds in the presence of 0.003% SDS. (C) GFP–Ras2p was inducibly expressed in wild-type yeast treated with compounds **4** or **8** at EC_5 concentrations. Cross-sectional fluorescence profiles were collected and used to calculate the relative amount of plasma membrane-localized fluorescence, which is graphed along with the standard error of the mean, as described in

Figure 1. (D) Increased dosage of *RCE1* restores normal GFP–Ras2p localization in compound treated cells. Wild-type yeast cells carrying either an empty plasmid or low-copy *RCE1* plasmid were treated with DMSO or 25 μM compound **4**, followed by induction of GFP–Ras2p expression. The strain used was EG123 (WT) and the plasmids were pRS416 (*CEN*) and pSM1314 (*CEN RCE1*)

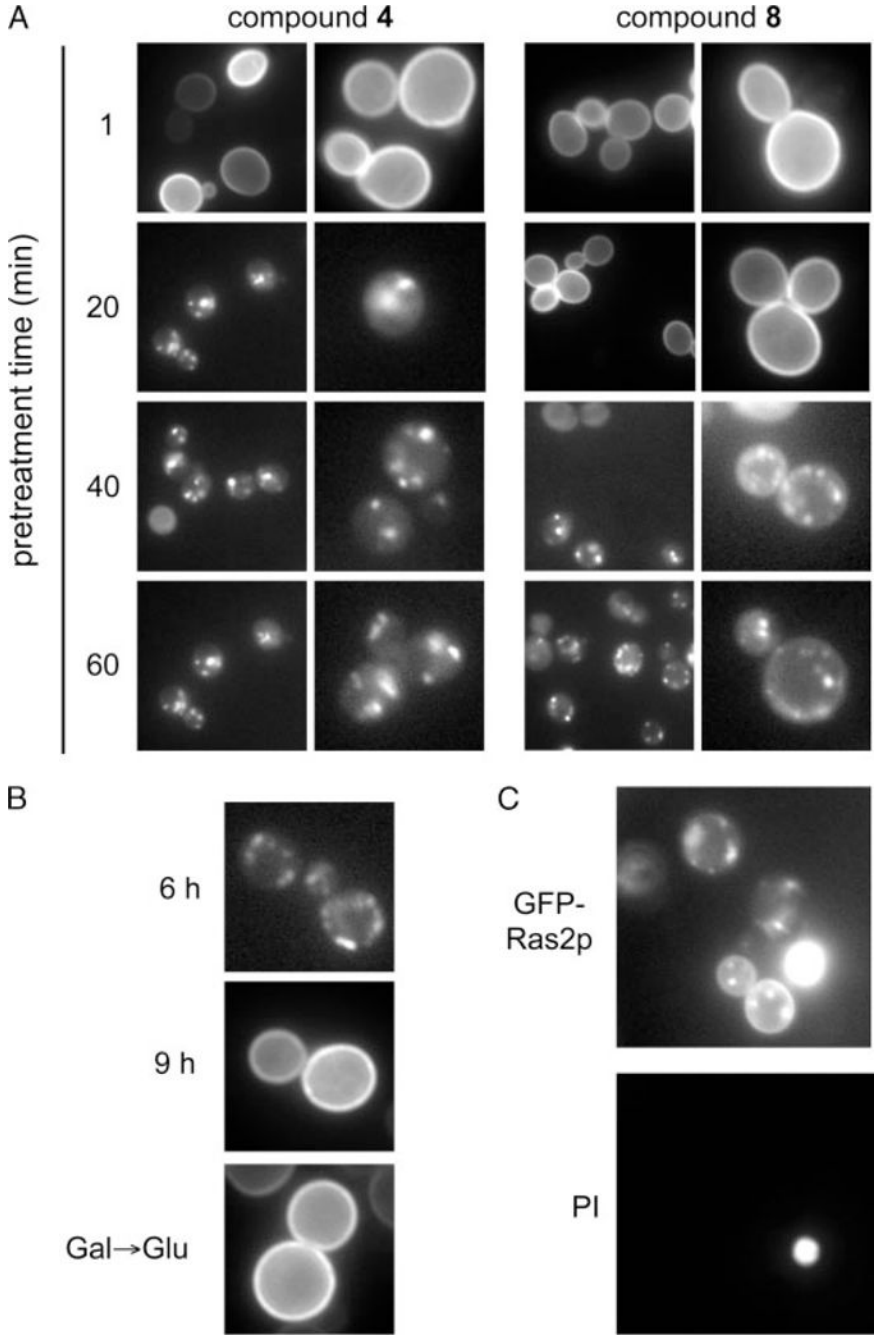

Figure 3.

Time-course studies of chemically induced GFP–Ras2p delocalization. (A) Compound-induced Ras delocalization is dependent on pretreatment time. GFP–Ras2p was inducibly expressed in wild-type yeast (EG123), as described in Figure 2, after pretreatment with the EC_5 concentration of compounds **4** or **8** for the indicated times. (B) Compound-induced delocalization is transient. Wild-type yeast (EG123) cells were compound treated and induced as described in Figure 2 (6 h), then a portion of cells was incubated in induction medium for an additional 3 h (9 h) or switched to glucose-containing medium and incubated for an additional 3 h (Gal → Glu). (C) Compound-induced delocalization of GFP–Ras2p

occurs in living cells. Wild-type yeast cells were pretreated with compound **8** and induced to express GFP–Ras2p, as described in Figure 2. After a 6 h induction period, the cells were stained with the nucleic acid dye propidium iodide (PI). The GFP and PI fluorescence signals observed for the same field of cells are shown. Within the cluster of cells shown, there are several live cells and one dead cell

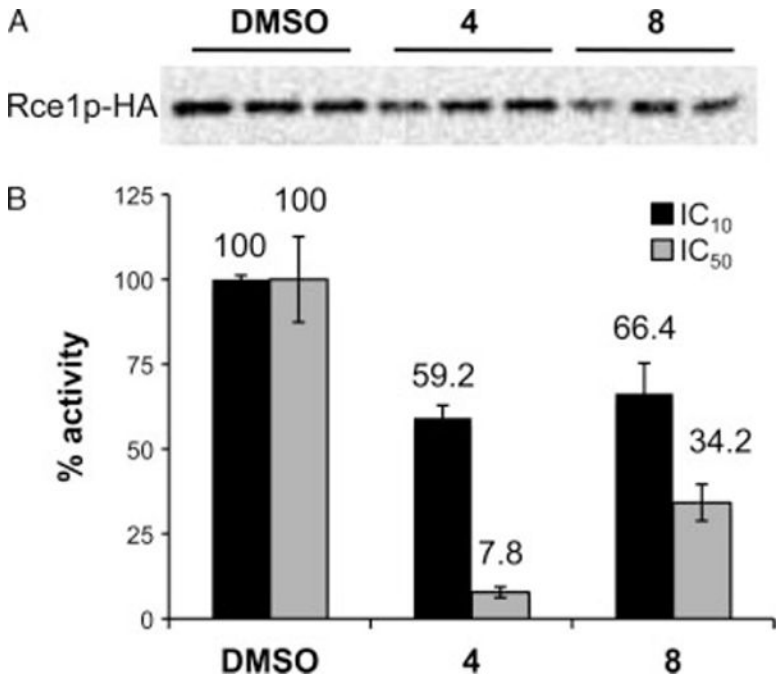

Figure 4.

Effect of *in vivo* compound exposure on expression and *in vitro* biochemical activity of yeast Rce1p. (A) *In vivo* compound exposure does not significantly alter Rce1p expression levels. Membranes were prepared from yeast over-expressing Rce1p that had been treated with DMSO or compounds **4** or **8** at their EC_{50} (61 and 97 μM, respectively) and EC_{10} (6.8 and 10.8 μM, respectively) concentrations; both treatment conditions yielded similar results, so only those associated with EC_{50} treatment are shown. Each treatment was performed in triplicate and independent membrane fractions recovered. An equal amount of total protein from each sample was analysed by SDS–PAGE and immunoblot, using an anti-HA antibody. (B) *In vivo* compound exposure reduces Rce1p activity in a dose-dependent manner. The membrane fractions described in (A), including those from the repeat experiment, were directly evaluated in duplicate for CaaX protease activity, using a fluorescent-based *in vitro* assay, yielding six (EC_{50}) or 12 data points (EC_{10}) for each individual treatment condition. These values were averaged, normalized to Rce1p expression levels, and are reported relative to the activity of the DMSO-treated condition. Statistical analysis was performed using Student's *t*-test, using the DMSO-treated condition as the reference; $p < 0.003$. The yeast used for Rce1p over-expression was strain SM3614, and the plasmid encoding Rce1p was pWS479

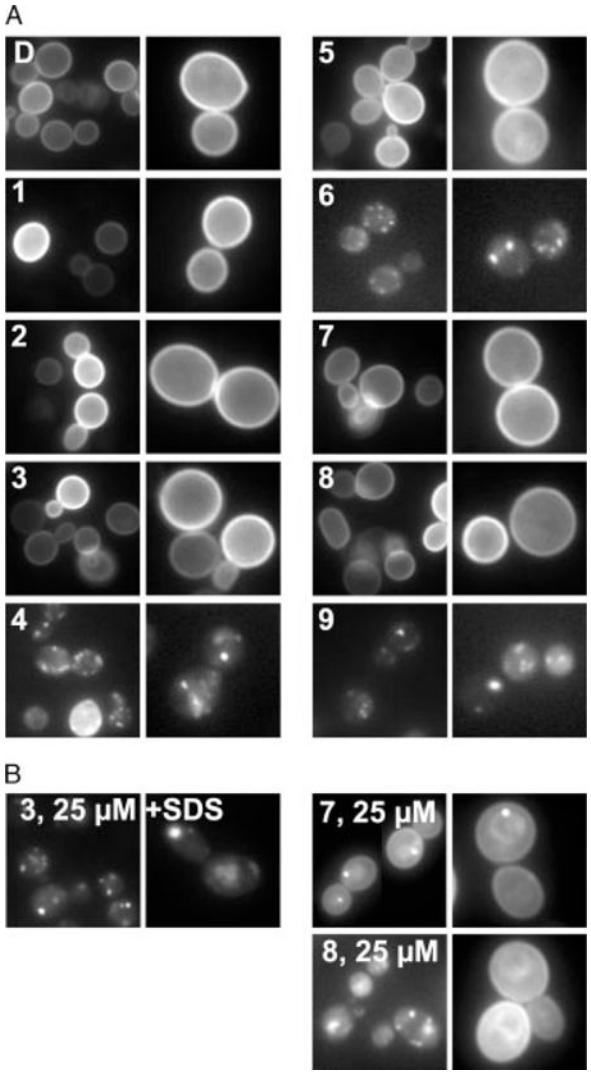

Figure 5.

GFP–Ras2p is an effective reporter for human Rce1p activity. GFP–Ras2p was inducibly expressed in yeast expressing human Rce1p as the sole source of CaaX proteolytic activity. Cells were pretreated for 1 h with or without the indicated compounds prior to induction as described in Figure 2. The strain used was yWS164 (*rce1*Δ *ste24*Δ) co-transformed with pWS335 (*HsRce1*) and pWS750 (*GFP–RAS2*). The concentrations used were EC_5 (A) or 25 μM (B)

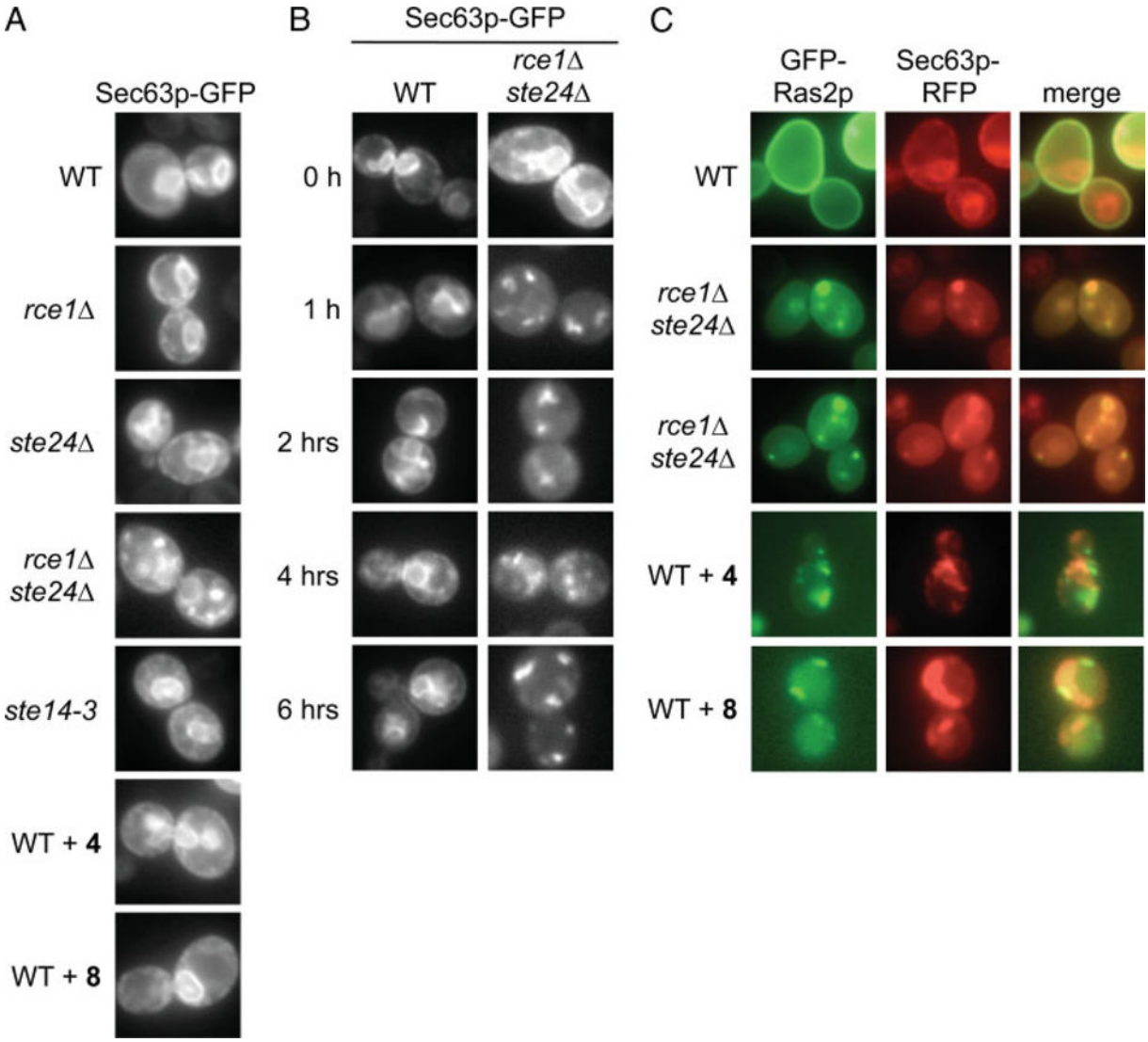

Figure 6.

The localization pattern of the ER-marker Sec63p–GFP depends on genetic background and nutritional status. (A) The distribution of Sec63p–GFP in yeast defective for the various steps associated with CaaX processing and in compound treated wild-type cells is relatively normal for cultures grown in glucose. The strains used were pJK59 transformants of the backgrounds described in Figure 1, with the exception of *ram1*Δ, which was not evaluated. (B) The distribution of Sec63p–GFP in wild-type (EG123) and CaaX protease-deficient (SM3614) yeast varies under conditions mimicking the protocol associated with GFP–Ras2p induction. Yeast were switched to galactose-containing media and images captured at the indicated times. (C) Co-localization of GFP–Ras2p (pWS750) and Sec63p–RFP (pSM1960) occurs under inducing conditions (galactose) only in the absence of CaaX proteolytic activity. The distribution of the indicated reporters in wild-type (EG123), CaaX protease-deficient (yWS164), and compound treated wild-type yeast was evaluated as described in Figure 2. Digital images were captured using either a fluorescein (left panel) or a rhodamine filter (middle panel) to capture GFP and RFP fluorescence, respectively. Merged images are

shown in the right panel. Where indicated, yeast were treated with compound **4** or **8** (each at 25 μM)

Table 1

Strains used in this study

Strain	Genotype	Reference
DF1	*MAT***a** *trp1 leu2 ura3 his4 can1 ram1::URA3*	(He *et al.*, 1991)
EG123	*MAT***a** *trp1 leu2 ura3 his4 can1*	ATCC No. 204 278
SM3103	*MAT***a** *trp1 leu2 ura3 his4 can1 ste24::LEU2*	(Fujimura-Kamada *et al.*, 1997)
SM3613	*MAT***a** *trp1 leu2 ura3 his4 can1 rce1::TRP1*	(Tam *et al.*, 1998)
SM3614	*MAT***a** *trp1 leu2 ura3 his4 can1 rce1::TRP1 ste24::LEU2*	(Tam *et al.*, 1998)
SM1188	*MAT***a** *trp1 leu2 ura3 his4 can1 ste14-3::TRP1*	(Hrycyna *et al.*, 1991)
yWS164	*MAT***a** *trp1 leu2 ura3 his4 can1 rce1::TRP1 ste24::KAN^R mfa1-Δ1 mfa2-Δ1*	(Cadiñanos *et al.*, 2003)

Table 2

Plasmids used in this study

Plasmid	Genotype	Reference
pJB1	*CEN URA3* P_{GAL}*-GFP-YCK2*	(Robinson *et al.*, 1999)
pJK59	*CEN URA3 SEC63-GFP*	(Prinz *et al.*, 2000)
pRS315	*CEN LEU2*	(Sikorski and Hieter, 1989)
pRS316	*CEN URA3*	(Sikorski and Hieter, 1989)
pRS416	*CEN URA3*	(Sikorski and Hieter, 1989)
pSM1107	*CEN URA3 HAn::STE24*	(Fujimura-Kamada *et al.*, 1997)
pSM1314	*CEN URA3 RCE1::HAc*	(Schmidt *et al.*, 1998)
pSM1960	*2* μ *URA3 SEC63-RFP*	Gift of S. Michaelis
pWS270	*CEN URA3* P_{GAL}*-GFP-RAS2*	(Manandhar *et al.*, 2007)
pWS335	*2* μ *URA3* P_{PGK}*-HIS::HA::HsRce1Δ22*	(Plummer *et al.*, 2006)
pWS479	*2* μ *URA3* P_{PGK}*-RCE1::HAc*	(Plummer *et al.*, 2006)
pWS750	*CEN LEU2* P_{GAL}*-GFP-RAS2*	This study
pWS751	*CEN LEU2* P_{GAL}*-GFP-YCK2*	This study
pWS808	*CEN URA3* P_{GAL}*-GFP-RAS1*	This study
pWS881	*CEN LEU2* P_{GAL}*-GFP-RAS1*	This study
YEp*RAS1*	*2* μ *LEU2 RAS1*	(Kataoka *et al.*, 1984)

Table 3

Summary of cytotoxicity and GFP-Ras2p delocalization observations

	Transient exposure				Continuous exposure
Compound[a]	**EC_{50} (μM)**	**EC_5 (μM)**[b]	**Growth at EC_5 (%)**	**Delocalization observed**[c]	**EC_{50} (μM)**
1	>500	26.3	109 ± 4	No	>500
2	>500	26.3	115 ± 2	No	>500
3	71	3.7	129 ± 6	Yes	7.90
4	61	3.2	110 ± 0	Yes	14.00
5	>500	26.3	108 ± 2	No	60.63
6	6	0.3	92 ± 6	Yes	0.07
7[d]	100	11.1	100 ± 8	Yes	0.07
8	97	5.1	104 ± 14	Yes	4.34
9	34	1.8	93 ± 6	Yes	0.08

[a] As previously reported (Manandhar *et al.*, 2007).

[b] Extrapolated from transient exposure EC_{50} dataset, assuming a Hill coefficient of 1; see Materials and methods for details.

[c] A positive score for delocalization after transient compound treatment required at least 50% of cells in the viewing area to have a discernible delocalization phenotype. Minimum treatment concentrations exerting an effect were EC_5 (compounds **4, 6–9**) or 25 μM (compound **3**; also required 0.003% SDS); other compounds (compounds **1, 2** and **5**) had no effect at the highest concentration evaluated (50 μM).

[d] Cell viability did not display a sigmoidal response to this compound.