Compartmentalized signaling of Ras in fission yeast

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Compartment-specific Ras signaling is an emerging paradigm that may explain the multiplex outputs from a single GTPase. The fission yeast, Schizosaccharomyces pombe, affords a simple system in which to study Ras signaling because it has a single Ras protein, Ras1, that regulates two distinct pathways: one that controls mating through a Byr2-mitogen-activated protein kinase cascade and one that signals through Scd1-Cdc42 to maintain elongated cell morphology. We generated Ras1 mutants that are restricted to either the endomembrane or the plasma membrane. Protein binding studies showed that each could interact with the effectors of both pathways. However, when examined in ras1 null cells, endomembrane-restricted Ras1 supported mating, and, conversely, plasma membrane-restricted Ras1 supported mating but did not signal to Scd1-Cdc42. These observations provide a striking demonstration of compartment-specific Ras signaling and indicate that spatial specificity in the Ras pathway is evolutionarily conserved.

Results and Discussion

Ras1 Palmitoylation Mutant Activates the Scd1 Morphology Pathway but Not the Byr2 Mating Pathway. Cysteine residues immediately upstream of CAAX sequences are palmitoylated in S. cerevisiae and higher metazoans. S. pombe Ras1, like S. cerevisiae Ras2p and mammalian N-Ras, contains a single such cysteine at position 215 (C215), and we have obtained evidence that it is palmitoylated (see Fig. 5, which is published as supporting information on the PNAS web site). To determine the functional significance of palmitoylation at position 215, we constructed expression vectors that use the ras1 promoter to express Ras1 or various Ras1 mutants, including one with a cysteine to serine substitution at position 215 to block palmitoylation. These vectors were integrated chromosomally in ras1 null (ras1Δ) cells via homologous recombination. Immunoblots were performed to screen for strains in which the exogenous Ras1 protein was expressed at a level similar to that of endogenous Ras1 (see Fig. 6, which is published as supporting information on the PNAS web site). Analysis of these strains revealed that whereas, as expected, wild-type Ras1 rescued both the mating and morphological defects, the palmitoylation-deficient mutant (Ras1C215S) rescued only the morphological defect (Fig. 1A). In contrast, the farnesylation-deficient mutant (Ras1C216S) and the double Ras1C215/216S mutant rescued neither defect, demonstrating that, unlike palmitoylation, farnesylation is absolutely required for all Ras1 functions. A palmitoylation-deficient, constitutively active Ras1 protein (8) (Ras1G17V,C215S) restored normal cell morphology without affecting the mating pheromone response (Fig. 1B), demonstrating that the selective rescue of the morphology pathway is not the result of inefficient GTP/GDP exchange. We have shown previously that yin6a in combination with ras1Δ creates a synthetic growth defect that results from the specific inactivation of the Cdc1-Cdc42 pathway (13). Fig. 1C shows that Ras1C215S, but

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Abbreviations: CFP, cyan fluorescent protein; ER, endoplasmic reticulum; GAD, Gal4 activation domain; PM, plasma membrane; RNC, C terminus of Rit; YFP, yellow fluorescent protein.

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not Ras1C215S or Ras1C216S, rescued the cold-dependent growth defect of yin6Δ ras1Δ cells, confirming the capacity of palmitoylation-deficient Ras to signal down the Scd1-Cdc42 pathway.

**Ras1C215S Can Efficiently Bind both Scd1 and Byr2.** To determine whether the substitution of serine for cysteine at position 215 of Ras1 affects the ability of the GTPase to bind to effectors, we tested Ras1-C215S for interaction with Byr2 and Scd1 by using a yeast two-hybrid assay. Ras1-C215S, like wild-type Ras1, bound both effectors with equivalent efficiency (Fig. 2A). In this assay, only the pool of the wild-type Ras1 fusion protein that enters the yeast nucleus can give a positive result. Although we do not have evidence that this pool is modified by palmitate, the critical interpretation of the result stands as follows: Palmitoylation is not required for the interaction of Ras1 with either Byr2 or Scd1. This conclusion is further supported by in vitro binding experiments in which GST-Byr2 pulled down, in a GTP-dependent fashion, equivalent amounts of wild-type Ras1, Ras1-C215S (Fig. 2B), and Ras1-C216S (Fig. 7, which is published as supporting information on the PNAS web site). Collectively, these two measures of protein-protein interaction suggest that the inability of Ras1-C215S to rescue mating was not because of an intrinsic defect in Byr2 binding.

**RasC215S Localizes to the Endomembrane but Not the PM.** We next determined the subcellular distribution of various Ras1 proteins

**Fig. 1.** Ras1C215S activates the Scd1, but not the Byr2, pathway. (A) ras1Δ cells expressing GFP-tagged versions of the indicated proteins from the endogenous ras1 promoter were imaged at either log phase (−) or after 3 days of starvation to induce sexual differentiation (+) and scored for sporulation (asterisks). (Scale bars: 5 μm.) (B) ras1Δ cells expressing GFP-tagged versions of the indicated proteins from the endogenous ras1 promoter were imaged at log phase. GFP-Ras1G17V, but not GFP-Ras1G17V,C215S, induced a hypersexual phenotype (B), marked by the presence of overextended conjugation tubes (arrowhead). (C) Cells lacking both ras1 and yin6 (ras1Δ yin6Δ) were transformed with vectors expressing the indicated GFP-tagged Ras1 proteins from the endogenous ras1 promoter. Transformed cells were serially diluted, spotted on plates, and incubated at the temperatures shown to determine whether various Ras1 proteins can rescue the cold-dependent growth defects of ras1Δ yin6Δ cells. Identical results were obtained in A–C whether Ras1 proteins were either untagged or tagged by a single copy of the hemagglutinin (HA) epitope and whether they were overexpressed by a high-copy vector.

**Fig. 2.** Various Ras1 mutants bind Byr2 and Scd1 with equal efficiency. (A) Protein-protein interactions were measured by the yeast two-hybrid system, and the results of activation of the HIS3 reporter gene are shown. Various Ras1 proteins were fused with the GAD. Byr2 was fused with the Gal4 DNA binding domain (GBD), whereas Scd1ΔN (containing the minimal Ras binding domain of Scd1) was fused with the LexA DNA binding domain (LBD). (B) His-Ras1, His-Ras1C215S (both with a T7 epitope tag) and GST-Byr2 were expressed and purified from E. coli. GST-Byr2 bound to glutathione-Sepharose beads was mixed with the GTP-γ-S- or GDP-loaded Ras1 proteins. Affinity purified proteins associated with Byr2 were then analyzed by immunoblot using an antibody directed to T7. (The binding of Byr2 to Ras1C216 was examined under identical conditions and is shown in Fig. 7.)
by expressing from the endogenous ras1 promoter GFP-tagged forms of these proteins in ras1Δ cells (Fig. 3A). As expected, GFP-Ras1 appeared on the PM, including that forming along the fission plane. Importantly, a weaker fluorescence signal was also observed on internal membranes. In contrast, GFP-Ras1C215S was not detected on the PM, and its localization on intracellular membranes was enhanced relative to that of wild-type Ras1. The latter pattern included the nuclear envelope and associated reticular structures and was similar to that of a GFP-tagged ER-resident protein, 13g6 (17). GFP-Ras1C216S did not associate with any membrane structure but rather appeared diffuse throughout the nucleoplasm and cytosol in a homogeneous distribution that revealed negatively imaged organelles.

To confirm this localization by an independent method, we performed subcellular fractionation. Whereas GFP-Ras1C216S was found entirely in the high-speed supernatant, both GFP-Ras1 and GFP-Ras1C215S were predominantly found in the membrane pellet (Fig. 3B), demonstrating that, although not localized to the PM, palmitoylation-deficient Ras1 is efficiently associated with other cellular membranes. Analysis of the membrane fractions by sucrose density gradient revealed that the peaks of endogenous Ras1 and 13g6 could be readily resolved with the Ras1 enriched fractions migrating further into the sucrose gradient, consistent with the behavior of PM-derived vesicles. Nevertheless, there was some overlap in the middle of the gradient, indicating that a portion of the total pool of endogenous Ras1 associated with lighter membranes, consistent with the fluorescent result (Fig. 3A). Endogenous Ras1 migrated in a pattern similar to that of GFP-Ras1. In contrast, the peak of GFP-Ras1C215S was shifted dramatically into the lighter membranes, and the pattern was nearly identical to that of membranes enriched for 13g6 (Fig. 3C). These results indicate that, whereas the bulk of Ras1 is in the PM, some remains associated with the endomembrane and that palmitoylation-deficient Ras1C215S is restricted to endomembrane. Thus, as in metazoan cells, Ras1 that is farnesylated but not palmitoylated is targeted to and accumulates on the ER.

**Fig. 3.** Ras1C215S localizes to the endomembrane. (A) Vectors expressing GFP-tagged forms of the Ras1 proteins from the endogenous ras1 promoter or the ER marker 13g6 (20) from the nmt1 promoter, were integrated into the chromosome of ras1Δ cells and imaged during log-phase growth. (Scale bar: 5 μm.) (B) High-speed supernatant (S) and membrane pellet (P) fractions of lysates from ras1Δ cells expressing the indicated proteins from the endogenous ras1 promoter were analyzed by immunoblotting with the GFP antibody. (C) Lysates from ras1Δ cells expressing either 13g6-GFP, GFP-Ras1, or GFP-Ras1C215S, or from wild-type cells expressing GFP alone, were applied to a 15–56% (wt/wt) sucrose gradient. After centrifugation, fractions were collected and analyzed by immunoblotting with either the anti-pan-Ras RAS 10 antibody or the GFP antibody.

**Ras1 at the PM Signals to the Byr2 Mating Pathway but Not the Scd1 Morphology Pathway.** Having thus demonstrated that ER-associated Ras1 signals down the Scd1, but not the Byr2, pathway, we sought to determine the functional capabilities of PM-restricted Ras1. To this end, we sought a C-terminal PM targeting motif that, unlike the CAAX motif, would not direct trafficking through the endomembrane. We reasoned that we should avoid the transmembrane tether of an intrinsic S. pombe PM protein because, as a secretory protein, it would transit the endomembrane en route to the PM and might therefore give an ambiguous result. Accordingly, we sought a protein that might be targeted directly from free polysomes in the cytosol to the PM. We examined the mammalian non-CAAX GTPase Rit that is targeted to the PM by a hydrophobic C terminus, which requires no posttranslational modification (18). Unlike GFP-H-Ras that, at steady state, localized to both the PM and Golgi apparatus, GFP-Rit localized exclusively on the PM (Fig. 4A). Indeed, GFP-Rit gave us the clearest PM targeting that we have observed to date. Moreover, by extending GFP with the C terminus of Rit (RitC), we showed that this region was necessary and sufficient for stringent PM targeting without any detectable endomembrane localization (Fig. 4A). We therefore made a cyan fluorescent protein (CFP)-tagged Ras1-RitC chimeric protein by replacing the C-terminal hypervariable domain and CAAX box of Ras1 with RitC (CFP-Ras1-RitC).

We coexpressed CFP-Ras1-RitC or CFP-tagged Ras1C215S in ras1Δ cells with a yellow fluorescent protein (YFP)-tagged 13g6 to mark the ER (Fig. 4B). All of the membranes marked by 13g6-YFP showed colocalization with CFP-Ras1C215S, indicating that palmitoylation-deficient Ras1C215S has significant affinity for the ER. CFP-Ras1C215S marked intracellular membranes, as well as cytoplasm, not labeled by 13g6-YFP. It should be noted that, as is the case with S. cerevisiae, much of the ER of S. pombe is found immediately subjacent to the PM such that electron microscopy...
is required for clear resolution of the two membrane compartments (19, 20) (see Fig. 8, which is published as supporting information on the PNAS web site), and decoration of the ER in these cells with fluorescent probes sometimes gives the appearance of PM at the resolving power of the light microscope. One distinction we have observed in the two similar patterns is that fluorescent probes that decorate the PM give a smooth linear pattern, whereas those that decorate ER give a lumpy pattern at the cell edge.

The distribution of CFP-Ras1-RitC in the PM was confirmed by subcellular fractionation on sucrose gradients that revealed CFP-Ras1-RitC to be enriched in heavier fractions (Fig. 4C). Like palmitoylation-deficient Ras1, Ras1-RitC bound both Scd1 and Byr2 with efficiencies equal to that of wild-type Ras1 (Fig. 2A). Importantly, functional analysis in ras1Δ cells revealed that, whereas GFP-Ras1C215S rescued the Scd1 morphology but not the Byr2 mating pathway, the converse was true of GFP-Ras1-RitC (Fig. 4D). Moreover, the inability of GFP-Ras1-RitC to rescue the growth defect of yin6Δ ras1Δ cells confirmed its deficiency in Scd1 signaling (data not shown). The inability of PM-restricted Ras1-RitC to signal to Scd1 suggests that it is the minor pool of endogenous Ras1 found on internal membranes (Fig. 3A), which signals down the morphology pathway.

Our results demonstrate that Ras1 restricted to the ER signals to the Scd1-Cdc42 pathway but is incapable of signaling down the

Byr2-mitogen-activated protein kinase (MAPK) pathway. The observation that constitutively active, endomembrane-restricted Ras1G17V,C215S also selectively activated the morphology pathway and demonstrates that compartmentalization occurs at the level of Ras1 rather than at the level of the GEF specific for this pathway. Conversely, Ras1 restricted to the PM signals via the Byr2-MAPK pathway but does not activate Scd1 and Cdc42. Because pheromone receptors relay mating signals across the PM, which activate Byr2, it seems appropriate that PM-associated Ras1 regulates this pathway. In support of this idea, Byr2 has been shown to associate with the PM upon sexual differentiation (21). We and others (12, 22) have shown that the Ras1-Scd1 pathway is involved in protein trafficking. Thus it is logical to expect that this pathway operates at the endomembrane. Consistent with this notion are the observations that mammalian Cdc42, when dissociated from RhoGDI, is localized predominantly on the endomembrane (23) and that the subcellular localization of S. pombe Cdc42 is indistinguishable from that of Ras1-C215S (24). Thus, downstream elements of the various Ras signaling pathways localize to the compartments upon which they are regulated by Ras and may therefore determine the spatial aspect of signaling.

In conclusion, the genetically tractable and relatively simple system offered by S. pombe has allowed a clear demonstration of compartmentalized Ras signaling. These results support the
observation in *S. cerevisiae* that one Ras effector, Eri1, which is a component of GTP-GlcNAc transferase, is restricted to the ER (25, 26). Our observations also support those observations reported in mammalian cells (27) and reveal that spatially restricted Ras signaling has been conserved through evolution.

Materials and Methods

*S. pombe* Strains and Microbial Manipulations. The wild-type strain used in this study is SP870 (11), *ras1Δ* (strains SPRU and SPRN) and *ras1Δ vin6Δ* (RAS1UYIN6K) strains were all derived from SP870 as described (9, 11, 13). Cells were grown in either yeast extract medium (YEAU) or synthetic minimal medium (MM) (28). To express genes controlled by the thiamine-repressible *nmt1* promoter, thiamine-free MM medium was used. All expression studies were done with vectors integrated into the *ars1* locus of the *S. pombe* chromosome, which was done by transforming cells with MluI-linearized vectors. All experiments were carried out with cells pregrown to early logarithmic phase (2–5 × 10^6^ cells/ml). To spot cells on plates, equal numbers of cells were diluted 1:5.

Mammalian Cell Culture and Transfection. Madin Darby canine kidney (MDCK) cells (American Type Culture Collection) were grown in DMEM containing 10% FBS (CellGro, Herndon, VA) at 5% CO_2_ and 37°C. All transfections (0.5 μg DNA per 35-mm dish) were performed 1 day after plating at 50% confluence by using SuperFectTM (Qiagen). Transfected cells were analyzed 1 day after transfection. To facilitate microscopy (see Microscopy), cells were imaged in the same 35-mm culture dish that incorporated a #1.5 glass coverslip with a sealed 15-mm cutout on the bottom (MatTek, Ashland, MA).

Plasmid Constructions. pSLF173, pGADGH, pVJL11, pLBDSCLΔN, pREPl, pARTCMRAS1, pARTCMRAS1G17V, pARTCMYIN6, and pGDB are as described (8, 9, 12, 13, 16, 29, 30). PREP4113G6GFP was a kind gift from the Candle laboratory (17). The pKH3RIT was generously provided by the Andres laboratory (University of Kentucky). pRPGFP and pRPFGPRAS1 carry the coding sequences for GFP and GFP-Ras1. The promoter in these plasmids was derived from 338 bp of the *ras1* 5' flanking sequence, which contains the *ras1* promoter (P. Papadaki and E.C.C., unpublished work). pTRCHISB was from Invitrogen. The Ras1C21SS, Ras1C216S, and Ras1C215/216S mutants were generated via PCR by using either pARTCMRAS1 or pARTCMRAS1G17V as the template. The forward primer used was 5'-CGGGATCCGATGAGGTCGCTTTTGAGACAT-3' and the reverse primers were 5'-CCGGATCCTCTAATAACAAGAGTTATTTG3-3' (for C215S), 5'-CCGGATCCCCTTAACTACATAACAGAAGTT-3' (for C216S) and 5'-CCGGATCCCTACCAATACATAACAAGAAGTT-3' (for C215/216S). The PCR products generated with the pARTCMRAS1 template were digested with BamHI and cloned into pEGFP-C1. The PCR products generated with the pARTCMRAS1G17V template were digested with BamHI and cloned into pEGFP-C1 and pEGFP-C1RIT. *ras1* was amplified by PCR using pARTCMRAS1 as a template, digested with BamHI, and ligated into pKH3RIT to generate pKH3RITC. To generate the Ras1-RitC chimera, a fragment encoding the last 62 C-terminal amino acids of the Rit protein was released from pKH3RIT by digesting with EcoRI and BamHI and ligated into pEGFP-C1 to form pEGFP-C1RITC. To generate the Ras1-RitC chimera, a fragment encoding the last 62 C-terminal amino acids of the Rit protein was amplified by PCR using primers 5'-CGGGATCCGATGAGGTCGCTTTTGAGACAT-3' and 5'-CGAAGGCTGGAGAGTTTTGAGACAT-3'. In addition, a fragment encoding a truncated form of Ras1 lacking its last 48 C-terminal amino acids was amplified by PCR using primers 5'-CGGGATCCGATGAGGTCGCTTTTGAGACAT-3' and 5'-CTAGCTCAGAGTTACACAGGG-3'. The forward and reverse primers were 5'-CCGGATCCGATGAGGTCGCTTTTGAGACAT-3' and 5'-GAAGATCTGTTTACTTGTACAG-3'. The PCR fragment encoding the truncated form of Ras1 was digested with BamHI and XbaI and then ligated into pBluescript SK– (Stratagene) to form pBSRAS1ΔC. The PCR fragment encoding the Rit C-terminus was digested with XbaI and SacI, and then ligated into pBSRAS1ΔC to form pBSRAS1ΔC. A BamHI-Sacl fragment encoding the Ras1-RitC chimera was released from pBSRAS1ΔC and ligated into pRPGFP to form pRPGFPΔC. To generate the CFP-tagged version of the Ras1-RitC chimera, a fragment encoding GFP was amplified by PCR using pECFP (Clontech) as a template. The forward and reverse primers were 5'-AATATGAAGTACAGTGGTTAAGAAACAGGCGAGGTACC-3' and 5'-CTAGCTCAGAGTTACACAGGG-3'. In addition, GFP was released from pRPGFP by digesting with PsiI and NheI to create pGFP. The PCR product encoding GFP was digested with PsiI and NheI and ligated into pRP to create pRCPFP. The BamHI-Sacl fragment encoding the Ras1-RitC chimera was then ligated into pRCPFP to create pRCPFPΔC. The BamHI-digested fragment encoding Ras1C21SS was ligated into pRPGFP to create pRCPFPΔC. To create 13g6 with a C-terminal GFP fusion, a fragment encoding 13g6 was amplified by PCR using pREP4113G6GFP as a template. The forward and reverse primers were 5'-CCGGATCCGATGAGGTCGCTTTTGAGACAT-3' and 5'-ATACACATGCGGCGGACAGTGTAAGTTTGACGAGGATG-3'. The 13g6 PCR product was then digested with XhoI and NotI and then ligated into pSLF173 to create pSLF13g6. In addition, a fragment encoding GFP was amplified by PCR using pEYFP (Clontech) as a template. The forward and reverse primers were 5'-ATAAGAATGCAGCTGGAAGACGGAAGACAGGGAAGGATGGTGACAG-3' and 5'-GAAGATCTGTTTACTTGTACAGTCTAGCCATGCAGG-3'. The PCR product was then digested with NotI and BglII and then ligated into pSLF13G6 to create pSLF13G6YFP. Expression of Various Ras1 Proteins at Endogenous Levels. *ras1Δ* cells (strain SPRN) were transformed with MluI-linearize pRPGFP vectors that express various Ras1 proteins off the endogenous *ras1* promoter. Transformed colonies were first selected to ensure that the linearized vector had been integrated chromosomally, and these clones of cells were further screened by Western blot using the pan anti-Ras monoclonal antibody RAS10 (1:500; Upstate Biotechnology, Lake Placid, NY) to seek cells in which the transgene was expressed at levels comparable to that of endogenous Ras1.

Yeast Two-Hybrid Assay. The binding was tested by measuring the activation of both the *lacZ* and *HIS3* reporter genes (11). Ras1 mutant proteins with abnormal C-termini as well as normal Ras1 proteins were fused with the Gal4 activation domain (GAD) as described earlier. Vectors expressing Byr2 and Scd1ΔN were as described (31, 32). pLBDSCLΔN autoactivates the *HIS3* reporter gene, so 3-AT (3-aminotriazole) was added to reduce background.
**GST Pull-Down Assay.** His-Ras1, His-Ras1C215S, His-Ras1C216S, and His-Ras1C215/216S were expressed in *Escherichia coli* BL21 DE3 cells and grown to midlog phase (6 × 10⁸ cells per ml) in 50 ml cultures of LB media containing both ampicillin and chloramphenicol. After induction with isopropyl-β-D-thiogalac- toside (IPTG) (Sigma), lysates were prepared in PBS buffer plus 1% Triton X-100 and phenylmethylsulfonyl fluoride (PMSF; Sigma). After treatment with DNase, crude lysates were centrifuged at 2,700 × g for 1 h. The reaction was terminated by placing the samples on ice and by adding 6 µl of 1 M MgCl₂. Supernatants were then incubated with 100 µl of His-Resin (Novagen) for 1 h at 4°C. Resin was washed with binding and wash buffers and incubated in elution buffer for 1 h at 4°C. GST-Byr2 was expressed using pGEX2TBYR2RBD (33), and lysates were similarly prepared from *E. coli*. Supernatants were incubated with 20 µl of glutathione Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C. Proteins were analyzed by immunoblot using the GFP antibody (1:500; Molecular Probes). Wild-type cells carrying vectors expressing either 13g6-GFP off the ras1 promoter, or GFP-Ras1C215S, or His-Ras1C215S off the endogenous ras1 promoter were grown in 100 ml of MM and lysed as described above. Cleared supernatants were applied to a 4.2-ml 15–56% (wt/wt) sucrose gradient prepared in lysis buffer. After centrifugation for 18 h (200,000 × g, 4°C), 300-µl fractions were collected from the bottom. Each fraction was analyzed by immunoblotting with the GFP antibody. The endogenous Ras1 was detected by the RAS10 monoclonal antibody.

**Subcellular Fractionation.** ras1Δ cells carrying integrated forms of vectors expressing either GFP-Ras1, GFP-Ras1C215S, or GFP-Ras1C216S off the endogenous ras1 promoter were grown in 100 ml of synthetic minimal medium (MM) to early log phase and 100 µl of 0.5 M EDTA and 1 µl of 10 mM GTPγS or GDP were added to collected from the bottom. Each fraction was analyzed by immunoblotting with the GFP antibody. The endogenous Ras1 was detected by the RAS10 monoclonal antibody.