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Rho2 Palmitoylation Is Required for Plasma Membrane Localization and Proper Signaling to the Fission Yeast Cell Integrity Mitogen-Activated Protein Kinase Pathway

Laura Sánchez-Mir,[a] Alejandro Franco,[a] Rebeca Martín-García,[b] Marisa Madrid,[a] Jero Vicente-Soler,[a] Teresa Soto,[a] Mariano Gacto,[a] Pilar Pérez,[b] José Cansado[a]

Yeast Physiology Group, Department of Genetics and Microbiology, Facultad de Biología, Universidad de Murcia, Murcia, Spain[a]; Instituto de Biología Funcional y Genómica, Consejo Superior de Investigaciones Científicas/Departamento de Microbiología y Genética, Universidad de Salamanca, Salamanca, Spain[b]

The fission yeast small GTPase Rho2 regulates morphogenesis and is an upstream activator of the cell integrity pathway, whose key element, mitogen-activated protein kinase (MAPK) Pmk1, becomes activated by multiple environmental stimuli and controls several cellular functions. Here we demonstrate that farnesylated Rho2 becomes palmitoylated *in vivo* at cysteine-196 within its carboxyl end and that this modification allows its specific targeting to the plasma membrane. Unlike that of other palmitoylated and prenylated GTPases, the Rho2 control of morphogenesis and Pmk1 activity is strictly dependent upon plasma membrane localization and is not found in other cellular membranes. Indeed, artificial plasma membrane targeting bypassed the Rho2 need for palmitoylation in order to signal. Detailed functional analysis of Rho2 chimeras fused to the carboxyl end from the essential GTPase Rho1 showed that GTPase palmitoylation is partially dependent on the prenylation context and confirmed that Rho2 signaling is independent of Rho GTP dissociation inhibitor (GDI) function. We further demonstrate that Rho2 is an *in vivo* substrate for DHHC family acyltransferase Erf2 palmitoyltransferase. Remarkably, Rho3, another Erf2 target, negatively regulates Pmk1 activity in a Rho2-independent fashion, thus revealing the existence of cross talk whereby both GTPases antagonistically modulate the activity of this MAPK cascade.

Protein S-acylation, also named protein palmitoylation, is a specific protein lipidation involving the thioesterification of selected cysteine residues within the target protein to the 16-carbon fatty acid palmitate (1). A number of proteins are palmitoylated *in vivo* in eukaryotes, from simple organisms like the yeast *Saccharomyces cerevisiae* to animal and human cell lines (2). Research on protein palmitoylation has drawn interest because of its regulatory and dynamic function and because some palmitoylated proteins are key players in the signaling mechanisms controlling cell proliferation, differentiation, and/or response to external stimuli (3). Prime examples of this control are members of the Ras and Rho family of small GTPases (4–7), which bind guanine nucleotides (GDP or GTP) and harbor intrinsic GTPase activity to hydrolyze the bound GTP (8). Guanine nucleotide exchange factors (GEFs) promote GTPase activation through dissociation and replacement of GDP by GTP (9). In addition, GTPase downregulation is exerted by GTPase-activating proteins (GAPs), which activate intrinsic GTPase activity by enhancing hydrolysis of GTP to GDP, and by GDP dissociation inhibitors (GDIs), which favor GTPase seizure to the cytosol in an inactive state (9).

Most Ras and Rho family GTPases undergo sequential lipid modifications for proper targeting to cellular membranes, which are essential for their biological activity (6, 10, 11). The first event of this sequence involves the covalent linkage of either farnesylpyrophosphate or geranylgeranylpyrophosphate to a cysteine residue located at a conserved C-terminal tetrapeptide motif known as the CAAX box (where A indicates an aliphatic residue and X is usually Ser, Met, Cys, Gln, Leu, or Ile) (2, 3). Then the AAX tripeptide is removed from the CAAX box by proteolytic cleavage, and the free carboxyl group of the isoprenylated cysteine is methylated by a specific isoprenylcysteine-*O*-carboxyl methyltransferase (12). However, in most cases additional motifs are needed to enhance and stabilize the membrane association of prenylated Ras and Rho GTPases. One is a cluster of polybasic amino acids located at a hypervariable region upstream of the CAAX box that favors electrostatic interaction with acidic membrane lipids (2). A second feature is the presence of one or two cysteine residues that become palmitoylated *in vivo* by a group of enzymes known as palmitoyltransferases (PTs), which locate to the endoplasmic reticulum and the Golgi region (3). Protein palmitoylation is a dynamic and reversible process, thus providing an attractive biological mechanism to compartmentalize both membrane targeting and signaling. Examples of the effects of palmitoylation dynamics on GTPase membrane sorting and function are the H- and N-Ras isoforms (6, 10) and Rho family small GTPases RhoB, TC10/RhoQ, and Rac1 (4, 5, 7).

The fission yeast *Schizosaccharomyces pombe*, a simple eukaryote whose signaling pathways show significant functional homology with those of higher cells (13), has a single Ras ortholog (Ras1), which is palmitoylated *in vivo* by Erf2 palmitoyltransferase (14, 15, 16). Notably, Ras1 signaling is spatially segregated so that cellular morphogenesis is regulated by an unpalmitoylated GT-

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Address correspondence to José Cansado, jcansado@um.es.
L.S.-M. and A.F. contributed equally to this article.
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