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Rce1: mechanism and inhibition

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Abstract

Ras converting enzyme 1 (Rce1) is an integral membrane endoprotease localized to the endoplasmic reticulum that mediates the cleavage of the carboxyl-terminal three amino acids from CaaX proteins, whose members play important roles in cell signaling processes. Examples include the Ras family of small GTPases, the γ -subunit of heterotrimeric GTPases, nuclear lamins, and protein kinases and phosphatases. CaaX proteins, especially Ras, have been implicated in cancer, and understanding the post-translational modifications of CaaX proteins would provide insight into their biological function and regulation. Many proteolytic mechanisms have been proposed for Rce1, but sequence alignment, mutational studies, topology, and recent crystallographic data point to a novel mechanism involving a glutamate-activated water and an oxyanion hole. Studies using *in vivo* and *in vitro* reporters of Rce1 activity have revealed that the enzyme cleaves only prenylated substrates and the identity of the a_2 amino residue in the Ca_1a_2X sequence is most critical for recognition, preferring Ile, Leu, or Val. Substrate mimetics can be somewhat effective inhibitors of Rce1 *in vitro*. Small-molecule inhibitor discovery is currently limited by the lack of structural information on a eukaryotic enzyme, but a set of 8-hydroxyquinoline derivatives has demonstrated an ability to mislocalize all three mammalian Ras isoforms, giving optimism that potent, selective inhibitors might be developed. Much remains to be discovered regarding cleavage specificity, the impact of chemical inhibition, and the potential of Rce1 as a therapeutic target, not only for cancer, but also for other diseases.

Keywords

Ras converting enzyme; CaaX proteins; Ras; proteases; cancer

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Introduction

Many eukaryotic proteins are isoprenylated with either farnesyl (C15) or geranylgeranyl (C20) groups. The CaaX-type prenyl proteins are historically defined by a carboxyl-terminal CaaX motif where C is cysteine, a is an aliphatic amino acid, and X is one of several amino acid residues. Proteins containing this motif are often subject to an ordered series of post-translational modifications (PTMs): C15 or C20 isoprenylation of the cysteine, endoproteolysis by Rce1 to remove aaX, and carboxyl methylation (Figure 1). Deviations from this standard pathway occur. Some proteins are isoprenylated, but not cleaved (i.e. shunt pathway), whereas other proteins are subject to additional PTMs after the canonical set of modifications (e.g. **a**-factor and *S*-acylation pathways). For the relatively few CaaX-type proteins examined in detail, disruption of the PTMs has profound effects on their function and localization. The CaaX-type proteins play an important role in cell signaling processes; their members comprise the Ras family of small GTPases, the γ -subunit of heterotrimeric GTPases, nuclear lamins, and protein kinases and phosphatases (Gao et al. 2009; Wang and Casey 2016). The CaaX proteins Ras, RhoA, RhoC, RheB, Rac proteins, CDC42, and PTP4A3 have been implicated in cancer (Winter-Vann and Casey 2005). Understanding the PTMs of CaaX proteins is therefore expected to provide insight into their regulation and biological function. This review focuses on the activity, mechanism, and inhibition of Ras converting enzyme (Rce1), which mediates the endoproteolytic cleavage of the carboxyl-terminal three amino acids from CaaX proteins.

Clinical relevance of Rce1

CaaX proteins are abundant eukaryotic proteins with diverse biological functions. They are involved in cancer, development, aging, parasitic growth, and many other biological pathways (Wang and Casey 2016). Ras GTPases are some of the more prominently studied CaaX proteins because of their involvement in cancer. Because Ras function optimally requires post-translational modification, there is intense interest for developing therapeutic inhibitors for all modification steps (Silvius 2002; Winter-Vann and Casey 2005; Konstantinopoulos et al. 2007; Berndt et al. 2011; Cox et al. 2015; Wang and Casey 2016). Such agents include farnesyl and geranylgeranyl transferase inhibitors (FTIs and GGITs), dual prenyl transferase inhibitors (DPIs), Rce1 protease inhibitors (RPIs), and carboxyl methyl transferase inhibitors (MTIs). FTIs are the most advanced of these agents and have reached clinical trials, but their utility as anti-cancer agents has not been substantiated (Gelb et al. 2006; Cox et al. 2015). The development and testing of RPIs and MTIs substantially lag that of prenyl transferase inhibitors. Rce1 is also being investigated as a potential biomarker in various types of cancer, but its utility as a disease predictor remains far from being understood (Huang et al. 2016; Li et al. 2017; Shi et al. 2017).

Studies investigating the importance of Rce1 in the cancer setting have largely relied on *rce1*^{-/-} mouse embryonic fibroblasts (MEFs; the mouse homozygous knockout is inviable), tissue-specific mouse knockouts, and small molecule agents that interfere with Rce1 activity (Chen 1998, 1999; Bergo et al. 2002, 2004; Wahlstrom et al. 2007). Loss of Rce1 activity is associated with Ras mislocalization, diminished growth of Ras-transformed fibroblasts in culture and in nude mice, and hypersensitivity to FTIs. Chemical inhibition of Rce1 yields

similar results. Collectively, these observations suggest that Rce1-deficiency correlates with a reduced ability to maintain the transformed state. It is unclear though whether outcomes observed with knockout approaches are directly due to defects in Ras signaling alone, or more likely, due to a broad impact of Rce1 inhibition on multiple CaaX proteins. The latter likely underlies the varied outcomes observed for tissue-specific knockout studies. Loss of Rce1 in hematopoietic tissue exacerbates K-Ras induced myeloproliferative disease (Wahlstrom et al. 2007). Loss of Rce1 in cardiac tissue results in high rates of lethal cardiomyopathy (Bergo et al. 2004). Loss of Rce1 in retina compromises photoreceptor function and is associated with retinal degeneration (Christiansen et al. 2011). Whether chemical-based inhibition of Rce1 will yield similar outcomes remains undetermined.

There is additional disease-related biology outside of cancer in which Rce1 is involved (Gelb et al. 2006). Rce1 is essential for growth of *Plasmodium* sp. and *Trypanosoma brucei*, the causative agents of malaria and African sleeping sickness, respectively (Gelb et al. 2003; Gillespie et al. 2007). Inhibitors that preferentially target parasite Rce1 over human could thus have potential clinical value in this setting (Mokry et al. 2009). In the case of certain bacterial infections, mammalian Rce1 regulates the modification and effectiveness of bacterial effector proteins injected into the eukaryotic host (Fueller et al. 2006; Price et al. 2010). Prokaryotic Rce1 itself appears to regulate the pathogenicity of bacterial species associated with high mortality rates, such as *Staphylococcus aureus* and *Streptococcus pneumoniae* (Frankel et al. 2010). Hence, the clinical importance of Rce1 may extend beyond cancer to several forms of infectious disease. Multiple developmental processes also appear to rely on Rce1. Loss of mouse Rce1 activity (i.e. *rce1*^{-/-}) results in late embryonic lethality, and tissue specific knockouts result in various disease states (Kim et al. 1999; Bergo et al. 2004; Wahlstrom et al. 2007; Christiansen et al. 2011).

Regulation of Rce1

Little is known about the regulation of Rce1 activity, and it is likely to have a general house-keeping function. Consistent with the widespread tissue expression of CaaX proteins, Rce1 is expressed in multiple tissues with some variation in expression levels (Otto et al. 1999; Young et al. 2001; Uhlen et al. 2015; Tissue Expression of RCE1, The Human Protein Atlas 2017). There is evidence, however, that Rce1 levels can be regulated by the USP17 deubiquitinating enzyme, whose activity leads to proteasomal degradation of Rce1 and hence reduced protein levels (Burrows et al. 2009; Jaworski et al. 2014). In wild-type MEF cells, the presence of USP17 results in down regulation of Rce1 activity, resulting in relocalization of GFP H-Ras from the plasma membrane to the cytosol and down regulation of Ras/MEK/ERK pathway signaling. Interestingly, these studies suggest that USP17 has differential impact on distinct splice variant isoforms of Rce1 in mammals (Rce1 Iso1 and Rce1 Iso2). Rce1 Iso2 protein levels appear to be regulated by USP17, whereas Rce1 Iso1 is unaffected. Nevertheless, both isoforms of Rce1 are required to reconstitute GFP H-Ras to the plasma membrane in *rce1*^{-/-} MEF cells, and USP17 on its own could not block cell proliferation in the absence of Rce1, suggesting a strong and direct link between the Rce1 and USP17 proteins. Further studies are required to understand the roles of different isoforms of Rce1 and their effect on the oncogenic Ras signaling pathway, as this would impact drug discovery efforts.

Identification and homologs of Rce1

The Ras converting enzyme (Rce1) is alternatively known as FACE-2 and Type II CAAX prenyl endopeptidase. Prior to its genetic identification, attempts to purify Rce1 using traditional biochemical approaches did not lead to its identification (Young et al. 2001). In retrospect, the main difficulties hindering the purification of Rce1 were its membrane-associated nature and the contaminating presence of Ste24p (FACE-1/Type I CAAX prenyl endopeptidase/Afc1), an evolutionarily unrelated protease with partially overlapping CaaX protease activity (Tam et al. 1998; Trueblood et al. 2000). The inability to biochemically purify or significantly enrich Rce1 is a complicating issue that continues to this date.

The genes encoding Rce1 and Ste24 were eventually both identified in *Saccharomyces cerevisiae* through an elegant genetic screen involving the **a**-factor mating pheromone as a reporter (Boyartchuk et al. 1997). Functional orthologs have subsequently been identified in other eukaryotic organisms (humans, mice, plant, parasite, worm, pathogenic fungi, etc.) (Kim et al. 1999; Otto et al. 1999; Cadiñanos, Schmidt, et al. 2003; Cadiñanos, Varela, et al. 2003; Mokry et al. 2009; Esher et al. 2016). Prokaryotic orthologs also exist, and while not expected to possess farnesyl-dependent specificity, at least one can cleave a synthetic farnesylated substrate *in vitro* (Pei and Grishin 2001; Manolaridis et al. 2013).

Collectively, Rce1 orthologs vary in their primary sequence identity (9–63%) and size (30–37 kDa) (Pei and Grishin 2001; Dore and Schmidt 2013). They all are predicted to be multi-span membrane proteins. Sequence divergence for transmembrane segments among homologs of any type is a common occurrence, and this may in part explain the low degree of sequence homology between Rce1 orthologs. Despite their low homology, Rce1 orthologs all have several conserved amino acids that are proposed to serve as catalytic residues: two adjacent glutamates and two separate histidines (Pei and Grishin 2001; Manolaridis et al. 2013). Other highly conserved residues include an arginine often positioned four residues away from the pair of glutamates, an aromatic residue (Phe or Tyr) often positioned four residues from the first histidine, and an asparagine/aspartate positioned near the second histidine. A cysteine near the second histidine is conserved in eukaryotic orthologs, but not in prokaryotic ones. The importance of conserved residues for the activity of eukaryotic Rce1 has been investigated for multiple eukaryotic orthologs, which are all expected to be involved in the cleavage of CaaX-type proteins (Dolence et al. 2000a; Plummer et al. 2006; Mokry et al. 2009). With exception of the proposed catalytic residues, the roles of other conserved residues are not yet fully understood and complicated by different observations. For example, the cysteine conserved in eukaryotic Rce1 orthologs has been reported to be essential for activity in one study but completely dispensable for activity in other studies (Dolence et al. 2000a; Plummer et al. 2006; Hildebrandt et al. 2013). Whether conserved residues are important for the activities of the prokaryotic orthologs remains an open question. Where examined, prokaryotic orthologs also appear to be associated with cleavage activities, including protection against antimicrobial peptides (Ellermeier and Losick 2006; Kjos et al. 2010). Rce1 has distinct, but somewhat similar sequence motifs to the PrsW family of proteases, the domain of unknown function protein DUF2324 from the Pfam family, and the 7-transmembrane-spanning APH-1 subunit of γ -secretase (Pei et al. 2011).

Topology and structure of Rce1

Rce1 is an integral membrane protein that is localized to the endoplasmic reticulum (ER) in eukaryotes (Schmidt et al. 1998; Bracha-Drori et al. 2008). Prokaryotic orthologs are envisaged to be located to the plasma membrane (Xiang et al. 2017). Hydrophathy and multiple-sequence alignment across Rce1 orthologs from *Homo sapiens* (*HsRce1*), *S. cerevisiae* Rce1 (*ScRce1*), and several other species predicted seven or eight trans-membrane segments depending on species (Figure 2) (Hildebrandt et al. 2013). Topology studies carried out on *ScRce1* and based on maleimide labeling of cysteines in *ScRce1* and in an HA tag placed at either the N- or C-terminal end of the enzyme suggested that the N-terminus projected into the ER lumen, whereas the C-terminus was located on the cytosolic side. This orientation was further supported by an N-linked glycosylation assay. Such an orientation would require an odd number of transmembrane spans (Figure 2(B)). Maleimide labeling of natural and introduced cysteines indicated that *ScRce1* might contain several reentrant helices, which do not span the bilayer, on the cytosolic face of the membrane (Figure 2(C)). The topology study relied heavily on mutants that introduced non-natural cysteines into *ScRce1*, which could introduce subtle changes that disrupt α -helices and helix hairpins leading to alternative topologies, misfolded protein, or both (Monne et al. 1999; Norholm et al. 2011). The maleimide reagent could also react with cysteines in large pockets that extend deeply into the membrane, which is a structural feature of Rce1 from an archaeon (vide infra).

The crystal structure of an Rce1 homolog from the archaea *Methanococcus maripaludis* (*MmRce1*) was elucidated at 2.5-Å resolution in complex with a monoclonal antibody Fab fragment (PDB accession number 4CAD) (Figure 3) (Manolaridis et al. 2013). Its structure, measuring approximately $35 \times 26 \times 46$ Å, is made up of 8 transmembrane α -helices linked by short loops plus two peripheral membrane α -helices (topology shown in Figure 2(A)). The C- and N-termini point into the ER lumen, which is in agreement with the hydrophathy predictions, but contradicts the topology data reported for *ScRce1* (Hildebrandt et al. 2013). At its core is a conical cavity with a large volume of 1400 \AA^3 that encompasses the catalytic site and opens to the cytosol. Inside the cavity, the conserved residues E140, E141, H173, H227, and N231 reside within the span of the membrane, and each project their side chains into the cavity, except E141, suggesting that it might not be directly involved in catalysis. The highly-conserved residue R145 sits at the base of the cone, separating the active site from the ER lumen. A water molecule is located in the cavity approximately 10 Å from the cytosolic surface of the membrane and is bridged by E140 and H173, which are positioned opposite one another. Additionally, one side of the cavity is open to the membrane via a gap between two of the transmembrane helices (TM2 and TM4).

Proteolytic mechanism

Early studies implicated Rce1 to be from the family of cysteine proteases based on the fact that mechanism-based cysteine protease inhibitors abolished activity and mutational studies showing that catalytic activity resulted when the only conserved cysteine among eukaryotic orthologs was changed to alanine (Ma et al. 1993; Chen et al. 1996; Dolence et al. 2000a). More recently, however, a study on *ScRce1* found that the only conserved cysteine among

eukaryotic Rce1 orthologs was not necessary for enzyme function, and a cysteine-free Rce1 mutant was fully functional *in vivo* (Plummer et al. 2006; Hildebrandt et al. 2013). Rce1 was also putatively assigned as a membrane-bound metalloprotease because of a conserved set of glutamates and histidines found across eukaryotic and prokaryotic orthologs (Pei and Grishin 2001). A mutational analysis study revealed the importance of these residues and the requirement of glutamate and histidine by *ScRce1* in catalysis, suggesting either a zinc metalloenzyme or a novel proteolytic mechanism (Plummer et al. 2006). Indeed, several pieces of data implicate a novel proteolytic mechanism, rather than the four established canonical mechanisms of proteolysis: aspartate, cysteine, serine/threonine, and metal-based. Rce1 has little sequence similarity with other proteases and no conserved cysteine across eukaryotes and prokaryotes (Pei and Grishin 2001). EDTA does not affect the activities of *MmRce1* or *ScRce1*, although excess Zn^{2+} inactivates *ScRce1*, but not *MmRce1* (Plummer et al. 2006; Manolaridis et al. 2013). Phenanthroline inactivation of *MmRce1* is the result of protein unfolding, and no Zn^{2+} was detected in *MmRce1* in proton-induced X-ray emission (PIXE) spectroscopy, total reflection X-ray fluorescence (TXRF) spectroscopy, or X-ray crystallography experiments.

The crystal structure of *MmRce1* suggested proteolysis through a novel mechanism, corroborating an earlier proposal (Plummer et al. 2006), involving an active site glutamate, two histidines, and an asparagine that are essential for catalytic activity (Figure 4) (Manolaridis et al. 2013). Computational docking studies, using a decapeptide containing the farnesylated canonical CaaX motif of RhoA (CLVS), suggested that the substrate adopts a β -hairpin structure to insert itself into the cavity. The farnesyl group fills the space between TM2 and TM4, which are decorated with non-polar amino acids, positioning the peptide bond between the cysteine and the first aliphatic amino acid near the water bridging H173 and E140 (Figure 3). The model does not provide insight into the position of the C-terminal carboxylate and any interactions that it might have with *MmRce1*. The water nucleophile attacks the carbonyl of the amide of the prenylated cysteine (Figure 5). H227 and N231, which form a structural element called an oxyanion hole, stabilize the resulting tetrahedral oxyanion intermediate that collapses to the carboxylate, jettisoning the amine, which is facilitated by proton transfer from H173 or E140, and completing the proteolysis. Although the proteolysis mechanism of Rce1 is novel, elements of the proposed mechanism, such as a glutamate-activated water and an oxyanion hole, are found in other membrane-associated proteases (Manolaridis et al. 2013): S2P (Feng et al. 2007), Ste24 (Pryor et al. 2013; Quigley et al. 2013), SGP (Fujinaga et al. 2004), and GlpG rhomboid protease (Baker et al. 2007; Ben-Shem et al. 2007; Wang and Ha 2007; Vinothkumar et al. 2010). The crystal structure model provides a foundation to gain coarse-level insight into the structure and mechanism of the eukaryotic Rce1; nevertheless, caution should be exercised with the mechanistic hypothesis because of species specific differences in the primary structure and topology of Rce1. For example, the primary structure of *MmRce1* is only 13% identical to that of *HsRce1* and 12% identical to *ScRce1* based on Clustal Omega analysis (UnitProtKB), and the N- and C-termini of *MmRce1* point into the ER lumen if adopting an *MmRce1* topology (Manolaridis et al. 2013), or are located on opposite sides of the ER membrane if oriented like *ScRce1* (Hildebrandt et al. 2013). Solved crystal structures of

human and yeast Rce1 would contribute significantly to a better understanding of the catalytic mechanism of proteolysis.

Reporter substrates for measuring Rce1 activity

Various reporter substrates have been developed to monitor Rce1 activity both *in vivo* and *in vitro* (Table 1). These reporters invariably take the format of an isoprenylated polypeptide with an aaX extension.

In vivo reporters

Many *in vivo* studies of Rce1 activity have relied on the yeast **a**-factor mating pheromone (36-mer polypeptide) and the genetic tractability that the yeast system provides. This includes the study that ultimately uncovered the identity of the *RCE1* gene (Boyartchuk et al. 1997). The **a**-factor reporter has since been used in various studies to systematically explore Rce1 and Ste24 specificity (Trueblood et al. 2000; Cadiñanos, Schmidt, et al. 2003; Cadiñanos, Varela, et al. 2003; Plummer et al. 2006; Bracha-Drori et al. 2008; Mokry et al. 2009). Through use of this reporter, there has been extensive but not exhaustive evaluation of CaaX protease specificity; collectively only ~75 CaaX combinations have been evaluated out of the 8000 possible CaaX sequence combinations (Boyartchuk and Rine 1998; Trueblood et al. 2000; Cadiñanos et al. 2003b; Young et al. 2005; Plummer et al. 2006; Krishnankutty et al. 2009; Mokry et al. 2009; Hildebrandt et al. 2016b). The utility of the **a**-factor reporter is tempered by the high sensitivity of the biological assays used to detect the reporter that could potentially lead to false-positives for cleavable CaaX motifs, an inability to distinguish between defective farnesylation and defective proteolysis for certain CaaX motifs, and the evaluation of CaaX motifs outside of their natural context. As an example, the CASQ motif has been widely regarded as an example of a Ste24-specific motif, but the amount of **a**-factor produced in the context of CASQ is only 1–2% relative to wild-type **a**-factor (CVIA), and the motif is not cleaved in its natural context as part of the yeast Hsp40 chaperone Ydj1 (Hildebrandt et al. 2016b). Despite these limitations, the **a**-factor reporter system has been one of the more useful genetic systems for exploring Rce1 activity and specificity.

By comparison to **a**-factor, Ras-based genetic methods are not widely used to study Rce1 activity due to their qualitative nature and reliance on rather complicated genetic set ups. Nevertheless, such methods have demonstrated that the absence of Rce1 modulates the thermosensitivity of yeast (Boyartchuk et al. 1997). More commonly, GFP-tagging of Ras and other Ras-related GTPases is used to assess the role of Rce1 in regulating small GTPase subcellular localization (Boyartchuk et al. 1997; Choy et al. 1999; Michaelson et al. 2005; Manandhar et al. 2007; Bracha-Drori et al. 2008; Roberts et al. 2008; Mokry et al. 2009; Hanker et al. 2010; Manandhar et al. 2010; Gentry et al. 2015; Mohammed et al. 2016). In the yeast system, GFP-Ras2 is cytosolic in the absence of farnesylation and has diffuse membranous patterns of intracellular distribution in the absence of either Rce1p or the ICMT (Ste14p) (Boyartchuk et al. 1997; Manandhar et al. 2010); the absence of Ste24p does not impact Ras2p localization. An altered response to heat shock is observed for the *rce1*

deletion relative to wild-type, suggesting that mislocalization of Ras disrupts the Ras signaling circuit (Boyartchuk et al. 1997).

In mammalian cells, Rce1-dependent localization is similarly observed for the H-, N-, and K-Ras isoforms (Choy et al. 1999; Kim et al. 1999; Bergo et al. 2002; Michaelson et al. 2005; Mohammed et al. 2016). The Rheb family of small GTPases also rely on Rce1 for their proper localization to the ER and Golgi (Hanker et al. 2010), as do the oncogenic Ras-like small GTPases RalA and RalB to the plasma membrane (Gentry et al. 2015). A reliance on Rce1 for proper localization of small GTPases is, however, not generalizable. It is often unclear whether perturbing Rce1 activity also impacts other associated post-translational modifications that occur to CaaX proteins (e.g. palmitoylation of H- and N-Ras (Hancock et al. 1990, 1991)). Rho-family proteins (i.e. Cdc42, Rac1, Rac2, and RhoA) are cytosolic, and this localization is independent of Rce1 activity (Michaelson et al. 2001, 2005). The localization of non-GTPase CaaX proteins can also vary in their dependency on Rce1 activity (Christiansen et al. 2011). Recently, GFP-Ras reporters have been useful for examining the impact of RPIs on Ras localization (Manandhar et al. 2010; Mohammed et al. 2016). Where examined, these inhibitors pharmacologically phenocopy effects observed with Rce1 knockouts and siRNA knockdowns. A major limitation of all localization studies, whether using knockouts or inhibitor-based approaches, is the possibility that altering the cleavage state of the entire prenylome at once may result in pleiotropic effects that confound interpretation of results. Moreover, delocalization effects can be difficult to quantify, especially when the signal is distributed across multiple locations. Despite these issues, GFP-based reporters continue to have great utility for studies of Rce1 and are potentially useful for future high-content HTS in association with inhibitor identification.

Recently, the yeast Hsp40 chaperone Ydj1p has been developed as yet another reporter, in this case one that is useful for both genetic and localization studies (Hildebrandt et al. et al. 2016b). Whereas the endogenous Ydj1p CaaX motif (i.e. CASQ) is subject only to isoprenylation and avoids subsequent PTMs, a process referred to as shunting, it can be made susceptible to Rce1 cleavage by mutation of the CaaX motif to those that are cleaved in the context of other reporters (e.g. CVIA and CTLM). Ydj1p thus has the potential to report on both cleavable and cleavage resistant CaaX motifs. Determining the specificity of Rce1 through use of various *in vivo* reporters is likely to help resolve how many CaaX proteins are shunted and how many are regulated by Rce1-mediated cleavage.

In vitro reporters

Rce1 *in vitro* activity can be monitored using a variety of farnesylated substrates, ranging from synthetic peptides modeled on CaaX proteins (e.g. Ras and a-factor) to recombinant proteins (e.g. GST-fusions) that have to be farnesylated *in vitro* prior to use. Early assays using synthetic peptides incorporated a radiolabeled amino acid within the aaX portion of the CaaX motif, enabling direct tracking of the liberated aaX tripeptide through HPLC and thin-layer chromatography methods [extensively reviewed in Young et al. 2001]. Indirect tracking of Rce1-mediated activity has involved use of synthetic peptide substrates or recombinant protein substrates, and coupled proteolysis-methylation assays. In these types of assays, ICMT-dependent methylation of the proteolyzed product is used to mark cleaved

substrates. Addition of a radioactive methyl group requires enrichment and quantification of the marked products, whereas addition of a non-radioactive methyl group to an **a**-factor-based reporter can be monitored directly using a biological assay (Tam et al. 2001).

A more utilitarian version of the direct assay monitors cleavage of an internally quenched fluorogenic (IQF) nonapeptide modeled on the farnesylated COOH-terminus of K-Ras4b (Hollander et al. 2000). In this assay, cleavage directly correlates with increased fluorescence, which is monitored in real time using a standard microplate reader having fluorescence detection capability. This assay approach is convenient for kinetic studies, HTS studies, and benefits from being non-radioactive. The assay confirms many of the expected properties of Rce1, ranging from isoprenoid dependence to tolerance for certain substitutions within the CaaX motif (Hollander et al. & Mallon 2003; Hildebrandt et al. 2016a). A major limitation of the IQF approach, however, is that either a fluorophore or quencher must be accommodated within the aaX portion of the CaaX motif, and such placement impacts Rce1 specificity and/or activity (Porter et al. 2007). For example, yeast Rce1 can tolerate lysine ϵ -dinitrophenyl (a quencher) at a_1 , and to a much lesser extent at X, but not at a_2 in the context of a farnesylated nonapeptide modeled on K-Ras4b; a similar observation has been made for human Rce1 (Porter and Schmidt, unpublished). The requirement of a non-natural amino acid within the aaX portion of the motif also limits the utility of this approach for Rce1 specificity studies since the entire combinatorial landscape of the CaaX motif cannot be explored. Nonetheless, the mix-and-measure nature of this assay continues to be useful and is a major reason that HTS-based small molecule RPI identification has been able to proceed (Manandhar et al. 2007).

Specificity of Rce1

Through use of the above *in vivo* and *in vitro* approaches to examine Rce1 activity, a snapshot of Rce1 sequence specificity is beginning to emerge. In terms of isoprenylation, Rce1 cleaves substrates modified with either C15 and C20, but not unprenylated substrates (Otto et al. 1999; Hildebrandt et al. 2016b). To the best of our knowledge, the specificity of Rce1 toward substrates with shorter and longer isoprenoids (e.g. C5 and C25) or alternative lipids (e.g. C14 and C16 acyl lipids) has not been examined. Earlier studies, where this topic might have been explored, would have also likely used membranes containing both Rce1 and Ste24 making specificity interpretations difficult. In terms of peptide length, a farnesylated tetrapeptide CaaX motif is the minimally effective length for Rce1 recognition, although Rce1 can also cleave certain isoprenylated tripeptides *in vitro* with kinetics that rival that of traditional farnesylated tetrapeptides (Hollander et al. 2003). The biological relevance of shorter motifs has not been explored *in vivo*.

In terms of amino acid specificity, the a_2 position appears to be the most critical residue for ScRce1 recognition as inferred from extensive substitution analysis of the **a**-factor CVIA motif (Trueblood et al. 2000). In this context, the most favored amino acids are Ile, Leu, and Val (i.e. branched chain, aliphatic amino acids). This is followed by Cys and Met, which are weakly tolerated. The next most critical residue appears to be a_1 for which there is more tolerance, with the best tolerances observed with Ala, Cys, Ile, Leu, and Val and the small polar residues Ser and Thr; some additional polar residues are weakly tolerated. These

results suggest that aliphatic is an oversimplification of allowable residues at the a₁ position. The traditional X position can be occupied by about half the amino acids without significant impact on Rce1p activity: Ala, Val, Ser, Thr, Asn, Gln, His, Phe, Tyr, Cys, and Met. Besides its high sensitivity, the **a**-factor-based assay used in the Trueblood study is subject to false-positive readouts. Another limitation is its coupled nature. Any sequence combination that reports negatively or weakly in the assay could result from incomplete farnesylation rather than incomplete proteolysis. Thus, Rce1 may tolerate other amino acids within the CaaX motif, but such substrates were not biologically available if incompletely farnesylated leading to a weak signal in the genetic-based assay.

Additional specificity information is likely to come from other assay approaches. These certainly could involve more comprehensive genetic screens involving **a**-factor to identify more cleavable CaaX combinations or Ydj1 to identify both cleavable and non-cleavable sequences, but these approaches will still be biased toward sequences that are efficiently farnesylated, or possibly even geranylgeranylated in some cases. Other reporters such as Ras could also be utilized. Intriguingly, the reported specificity preferences of ScRce1 observed with the **a**-factor reporter are similar to those inferred from a Ras-based yeast genetic screen intended to interrogate farnesyl transferase specificity (Stein et al. 2015). The Ras-based screen evaluated the farnesylation potential of all 8,000 possible CaaX variations. Analysis of the top performing sequences reveals that a₂ is enriched for Ile, Val, Leu, Met, and Cys (Figure 6). Thus, both Ras and **a**-factor reporters identify similar enrichment of amino acids at a₂; considerable variation was observed at a₁ and X. It is tempting to speculate that the top performing sequences in the Ras-based study are cleaved, but this was not directly assessed. Moreover, the Ras-based screen was conducted in the presence of both Rce1 and Ste24, making it unclear which protease is responsible for cleavage of the top Ras-based sequences. The Ras-based assay also suffers from the same limitation as the **a**-factor based assay in that incompletely farnesylated sequences could report as false-negatives in the Ras-based assay despite having amino acids compatible for cleavage. For a truly unbiased identification of Rce1 cleavable sequences, it is feasible to expect that a synthetic library representing 8000 farnesylated Cxxx tetrapeptides could be developed to explore specificity without concern for isoprenylation status. Such an approach currently has technical and cost-related challenges.

Inhibitors of Rce1

Inhibitors of Rce1 that are not only potent but also selective could be used to further investigate the physiological role of Rce1 in regulating CaaX proteins and downstream effects of its inhibition. There are a number of Rce1 inhibitors reported in the literature (Bergman et al. 2011; Schmidt and Dore 2011; Dore and Schmidt 2013; Mohammed et al. 2016) that can be categorized as follows: anti-sense reagents to target the Rce1 mRNA, general protease inhibitors, substrate mimetics, and small molecule inhibitors.

Anti-sense reagents

A cocktail of three siRNA oligonucleotides (19 bases) knocks down human Rce1 expression in human colon carcinoma (HCT-116) cells (Mohammed et al. 2016). A 60–75% reduction

in Rce1 mRNA levels occurred with a mixture of siRNA oligonucleotides targeting a translated sequence (GUCAUCAAGCGACGCUUCA), the end of the translated region (GCUCCUGACCUAUGCUCU), and a 3'-untranslated section of the mRNA (CCUCUGCCUCU GAAAAGCU). Treatment of HCT-116 cells expressing GFP tagged H-, N-, or K-Ras (K-Ras4b) with the siRNA cocktail also mislocalized the Ras isoforms. N-Ras was most affected followed by H-Ras, then K-Ras4b. While not directly inhibiting Rce1 activity, siRNA represents a powerful tool to explore the downstream impact of Rce1 function.

General protease inhibitors

The general inhibitors cover a variety of structures. These include chloromethyl ketones such as N_{α} -tosyl-L-phenylalanine chloromethyl ketone (TPCK), which is considered a non-specific irreversible inhibitor of serine/cysteine proteases (Figure 7). This inactivates *ScRce1* with an approximate K_i of 1mM (Chen et al. 1996). Interestingly TPCK inhibits *ScRce1* in the presence of a Ras reporter, but not in the presence of the α -factor peptide. Therefore, it should be noted that differential inhibition of Rce1 can depend upon the reporter utilized in biochemical activity assays. TPCK has comparable inhibitory activity against Rce1 from species other than yeast, including *Arabidopsis thaliana* (*AtRce1*), human (*HsRce1*), and trypanosome (*TbRce1*) (Porter et al. 2007; Mokry et al. 2009). When applied at 250 μ M, TPCK inhibited *ScRce1*, *AtRce1*, and *HsRce1* to 32, 0.17, and 7%, respectively. A similar 200- μ M concentration of TPCK inhibited *TbRce1* activity to 22% (Mokry et al. 2009). A more lipophilic chloromethyl ketone, N_{α} -tosyl-L-lysine chloromethyl ketone (TLCK), is a trypsin and trypsin-like enzyme inhibitor. By comparison to TPCK, TLCK does not inhibit Rce1 under the same conditions. Other examples of general protease inhibitors reported to inhibit Rce1 include the organomercurial compounds *para*-hydroxymercuribenzoic acid (pHM), *para*-hydroxy-mercuriphenylsulfonic acid (pHMS), and mersalyl acid (MSA) (Dolence et al. 2000a). Non-specific organomercurials and other general protease inhibitors have generally not been useful for probing the proteolytic mechanism of Rce1 and have led to various erroneous proposed mechanisms for Rce1.

Substrate mimetics

A number of inhibitors can be justifiably classified as substrate mimetics based on structural elements that mimic the natural substrate features. The first specific, non-reversible inhibitor of Rce1 was created by modifying TPCK and TLCK with a farnesyl group and replacing the sulfonamide with a *tert*-butylcarbamate to create BFCCMK ($K_i = 30 \mu$ M) (Figure 8) (Chen et al. 1996). Replacement of the thiofarnesyl unit with a tridecyl group gave a more stable and cell-permeable analog, UM96001 (Chen 1998).

Peptidyl acyloxymethyl ketones (AOMKs) inhibit Rce1 (Porter et al. 2007). The peptidyl portion modulates the inhibitory properties of these compounds, but significant improvement of inhibitory properties is not likely achievable through alteration of the peptidyl portion alone (Dechert et al. 2010) (Figure 8). Because AOMKs are known to be irreversible cysteine protease inhibitors, the reactivity of these derivatives can also be modulated by altering the acyloxy leaving group. Various derivatives with differing benzyloxy groups have been evaluated, but AOMKs surveyed had weak inhibitory properties against *ScRce1* (>40%

ScRce1 activity remaining after 100- μ M treatment). A substituted analog that did not contain a leaving group suitable for an irreversible binding mechanism (“warhead-free” AOMK, Figure 8) proved to be the best inhibitory compound (~25% *ScRce1* activity remaining after 100- μ M treatment). These results suggest that AOMKs do not likely work as mechanism-based inhibitors and are most likely non-covalent, reversible inhibitors of *Rce1*.

To date, the most potent of all *Rce1* inhibitors are the farnesylated peptide mimetics, RPI and a statine analog (SA) (Figure 9) with $K_i = 86$ and 64 nM, respectively (Ma et al. 1993; Chen et al. 1996), and an IC_{50} value of 5 nM for RPI (Otto et al. 1999). RPI and SA are farnesylated CaaX motifs with a non-hydrolyzable linkage between cysteine and the aaX sequence of K-Ras (CVIM). SA is most likely a transition state analog inhibitor of *Rce1*. RPI and SA remain the gold standard in terms of inhibitory activity against *HsRce1*. In addition to RPI and SA, other tetrapeptide inhibitors (peptides 1–5, Figure 9) have been developed. Many of these are less potent by comparison to RPI and SA (*ScRce1* $IC_{50} = 3.3$ –17.6 μ M) (Dolence, Dolence, et al. 2000; Dolence, Steward, et al. 2000; Dolence et al. 2001).

Non-peptidyl, non-prenyl inhibitors (NPNPIs) of the farnesyl transferase also inhibit *HsRce1* (Schlitzer et al. 2001). The three best compounds NPNPI-A, B, C (Figure 10) displayed moderate inhibitory activity with IC_{50} values of 14, 7, and 7 μ M, respectively. These are bi-substrate analogs that contain a peptidomimetic representing the CaaX motif and a lipophilic non-prenyl moiety representing the farnesyl group. The fatty acid lipid-like structure of these inhibitors invites speculation that these might be promiscuous aggregate-based inhibitors (McGovern et al. 2002 2003; Coan and Shoichet 2008).

The marine natural products barangcadioic acid A and rhopaloic acids A to G (Figure 11), isolated from *Hippospongia* species, are terpenoids with IC_{50} values of approximately 26 μ M in an *HsRce1*-proteolysis assay (Craig et al. 2002). These are the first reported natural product inhibitors of *Rce1*. Akin to better performing *Rce1* inhibitors, these natural products all carry an isoprenoid moiety. Additionally, extracts from the Indonesian sponge *Carteriospongia foliascens* contained two already known and four previously unreported scalarane-based sesterterpenoids (Williams et al. 2009). Of the six compounds, a mixture of two 20,24-bishomoscalarane ketals, differing in the stereochemistry at C-24, inhibited activity of *HsRce1* ($IC_{50} = 7.1$ μ M) despite the fact that these derivatives do not have an isoprenoid group (Figure 11).

Small molecule inhibitors

Recent work to identify new inhibitors of *Rce1* has focused on the development of non-peptide based structures in an effort to identify small, drug-like molecules. Such compounds would be expected to have improved cell penetration and easier synthetic accessibility compared to peptidomimetic and natural compound-based inhibitors, enabling the use of eukaryotic cell-based assays of *Rce1* activity, which would aid investigation into the effects of inhibiting *Rce1* at the cellular level.

The first set of small molecule *Rce1* inhibitors reported in the literature were identified as inhibitors of *ScRce1* in a medium throughput screening campaign using a National Cancer

Institute (NCI) library of approximately 2000 compounds (Manandhar et al. 2007). A final set of nine compounds demonstrated IC₅₀ values of 6–35 μM against *ScRce1*. The specificity of the compounds was evaluated in part by examining their inhibitory properties toward other proteases. All of the compounds inhibited *ScSte24* (IC₅₀ = 8–28 μM), but had little or no activity on trypsin at ~30 μM. For *HsRce1* and *TbRce1*, IC₅₀ values of 0.4–46 and 0.25–177 μM were observed, respectively (Mokry et al. 2009). Four of the compounds were complexes with toxic metals (mercury, palladium, or copper), and one was santowhite powder, an antioxidant added to rubber and latex. The additional four hits were NSC609974, NSC73101, NSC1011, and NSC270718 (Figure 12). In a subsequent yeast study, NSC1011 and NSC73101 disrupted the proper membrane localization of a GFP-Ras2p reporter (Table 1) (Manandhar et al. 2010). In yeast expressing *TbRce1* instead of *ScRce1*, six of the nine compounds disrupted GFP-Ras2p membrane localization, including NSC1011. No species-specific selectivity was observed for the trypanosomal protein over the human ortholog, however, which diminishes enthusiasm for selective targeting of *TbRce1* in the search for therapeutic compounds.

NSC609974, NSC73101, and NSC270718 were active in other screening programs, suggesting that these might be pan-assay interference compounds (PAINS) (Baell and Holloway 2010; Thorne et al. 2010; Baell et al. 2013; Baell and Walters 2014; Baell 2016). NSC 609974, also known as carboxyamidotriazole (CAI) or L651582, inhibits several calcium-mediated signal transduction pathways (Kohn and Liotta 1990; Felder et al. 1991; Kohn et al. 1992). It is an inhibitor of receptor-operated non-voltage-gated and voltage-gated calcium influx (Felder et al. 1991; Hupe et al. 1991), calcium-dependent arachidonic acid release (Clark et al. 1991; Felder et al. 1991), and tyrosine phosphorylation of phospholipase Cγ (PLCγ) (Gusovsky et al. 1993), and it has been used to study how calcium influx modulates the expression of matrix metalloproteinase-2 (MMP2) (Kohn et al. 1994b). NSC609974 and its orotate salt possess anti-proliferative, anti-angiogenic, and anti-metastatic properties (Kohn et al. 1992; Kohn, Felder, et al. 1994a; Mignen et al. 2005; Guo et al. 2006; Corrado et al. 2012) and have been in clinical trials for cancer (Grover et al. 2007; Taylor et al. 2015; ClinicalTrials.gov 2017). It is tempting to speculate that the anti-oncogenic activity and perhaps some of the adverse side effects could be due in part to the inhibition of Rce1.

NSC73101 was identified in a screen for inhibitors of poxvirus type I topoisomerase, but it was not the most promising inhibitor and was not pursued further (Bond et al. 2006) It was also identified in other screens as an inhibitor of *Vibrio cholera* biofilm formation (Peach et al. 2011), an inhibitor of the manganese/magnesium-dependent phosphatase PP2Cα (Sierecki and Newton 2014), and an irreversible inhibitor of replication protein A, an essential DNA repair protein (Gavande et al. 2016).

NSC270718 was independently found in multiple HTS and virtual ligand docking screens to be a moderate inhibitor of angiogenin ($K_i = 75 \mu\text{M}$), an inducer of new blood vessel growth (Jenkins et al. 2002; Kao et al. 2002); the human mitotic kinesin Eg5 (DeBonis et al. 2004); DNA synthesis specific to Kaposi's sarcoma-associated herpes virus (KSHV) (IC₅₀ = 2.38 μM) (Dorjsuren et al. 2006); the soluble form of adenylyl cyclase (Schlicker et al. 2008), and the ATP-dependent MurD ligase (IC₅₀ = 34 μM in biochemical assays), an enzyme involved

in the biosynthesis of an essential peptidoglycan component of the bacterial cell wall (Turk et al. 2009). It was also identified in the same screen against poxvirus type I topoisomerase that NSC73101 was found (Bond et al. 2006).

Other biological activities for NSC1011 have not been reported, but its structural analog, NSC66811 (Figure 13) inhibits the interaction between murine double minute 2 (MDM2) and p53 tumor suppressor with a $K_i = 120$ nM (Lu et al. 2006). The oncogene MDM2 is the cellular inhibitor of p53, which is an essential component of cell cycle regulation. In part because of this relatively higher potency against *HsRce1*, NSC1011 was taken forward into an SAR study in an attempt to improve upon potency and selectivity toward *HsRce1* (Mohammed et al. 2016). A small library of approximately 56 new compounds was synthesized around the core 8-hydroxychloroquine scaffold (Figure 13) and tested against *HsRce1* using the *in vitro* IQF proteolysis assay. Many of the derivatives failed to improve upon potency, although substitutions on the benzoic acid and phenyl rings were well-tolerated. Changing the hydroxyquinoline ring to a 1-naphthol moiety showed a modest improvement in IC_{50} against *HsRce1* from 6.9 μ M for NSC1011 to 4.2, 3.9, and 3.8 μ M for compounds **29**, **31**, and **46**, respectively (Figure 13). The hydroxyl group on the quinoline was essential for biological activity. The most active compounds were selective for *HsRce1* versus *HsSte24*, and a representative subset of NSC1011 analogs did not inhibit human farnesyl transferase. NSC66811 was not active against *HsRce1*.

The effectiveness of NSC1011-based compounds to work *in vivo* was demonstrated through a Ras mislocalization assay. NSC1011 and compounds **2**, **5**, **6**, and **17** were effective at mislocalizing Ras to the cytosol in a human colorectal carcinoma cell line (HCT-116). Interestingly, the Rce1 inhibitors mislocalized N-Ras to the greatest extent, followed by H-Ras, then K-Ras4b. A similar result was found using Rce1 siRNA knockdowns (Mohammed et al. 2016). Curiously, NSC1011 and its analogs were more effective than an FTI at mislocalizing K-Ras4b, which is significant, because activating K-Ras mutations are more frequently observed in cancer compared to those of H- and N-Ras (Prior et al. 2012).

Perspectives

Rce1 has been studied for more than two decades. A great deal of progress has been made in terms of elucidating the precise involvement of Rce1 in CaaX protein maturation, yet much remains to be discovered regarding its cleavage specificity and impact of chemical inhibition, especially *in vivo*. Efforts to date have focused on three main areas: genetic inhibition to identify the impact of down regulation of Rce1, structural elucidation of a distantly related archeal ortholog, and development of chemical probes for its inhibition. Future inhibitor development is certainly currently limited by the lack of structural data of a eukaryotic enzyme. This has made it difficult to understand the mechanistic properties of existing inhibitors and has delayed structure-based design of inhibitors that may have better inhibitory properties than those currently available.

Whereas the current most potent inhibitor of Rce1 is a farnesylated tetrapeptide (i.e. a likely transition state substrate mimetic), it is cell impermeable and not useful to investigate the effects of Rce1 inhibition *in vivo*. A crystal structure involving the complex of SA with Rce1

should be a high priority. If it is a transition state inhibitor, then the hydroxyl group should displace the catalytic water molecule in the active site. The activity of the hydroxy epimer of SA against Rce1 would also be of interest to give insight into the orientation of the substrate in the active site. Attempts to overcome the issue of membrane impermeability of RPI and SA through development of small molecules are beginning, but these compounds have so far fallen short of expected standards for detailed animal studies. Nevertheless, the small molecule inhibitors identified thus far with activity in both *in vitro* and cell-based assays suggest that potent and selective inhibitors might yet be developed. New scaffolds may derive from additional HTS screening and possibly through computational methods for structure-guided design. Regardless of how developed, understanding the inhibitory mechanisms of these new compounds will undoubtedly require new structures of Rce1 orthologs, especially a eukaryotic one, and preferably that of the human enzyme.

Once appropriate inhibitory compounds are developed, it will be interesting to investigate whether Rce1 inhibitors alone or in combination with FTIs will be more effective than FTIs alone at modulating Ras activity. It is intriguing to speculate that modulation of K-Ras4b activity, in particular, might be more effective when FTIs are applied in combination with Rce1 inhibitors. It is well documented that alternative geranylgeranylation of K-Ras4b occurs in the presence of FTIs. Concomitant use of FTIs and Rce1 inhibitors could thus help limit the residual function associated with geranylgeranylated K-Ras4b. Inhibitors, regardless of their impact on Ras activity, may also have overlooked utility in other disease situations. Many CaaX-type isoprenylated proteins exist, and they are involved in many cellular processes. It is not yet clear, however, which CaaX proteins are cleaved by Rce1 as opposed to Ste24. Understanding the specificities and mechanisms of both enzymes is thus intertwined for developing accurate substrate profiles, which will be needed to better understand the potential biological impact of Rce1 inhibition.

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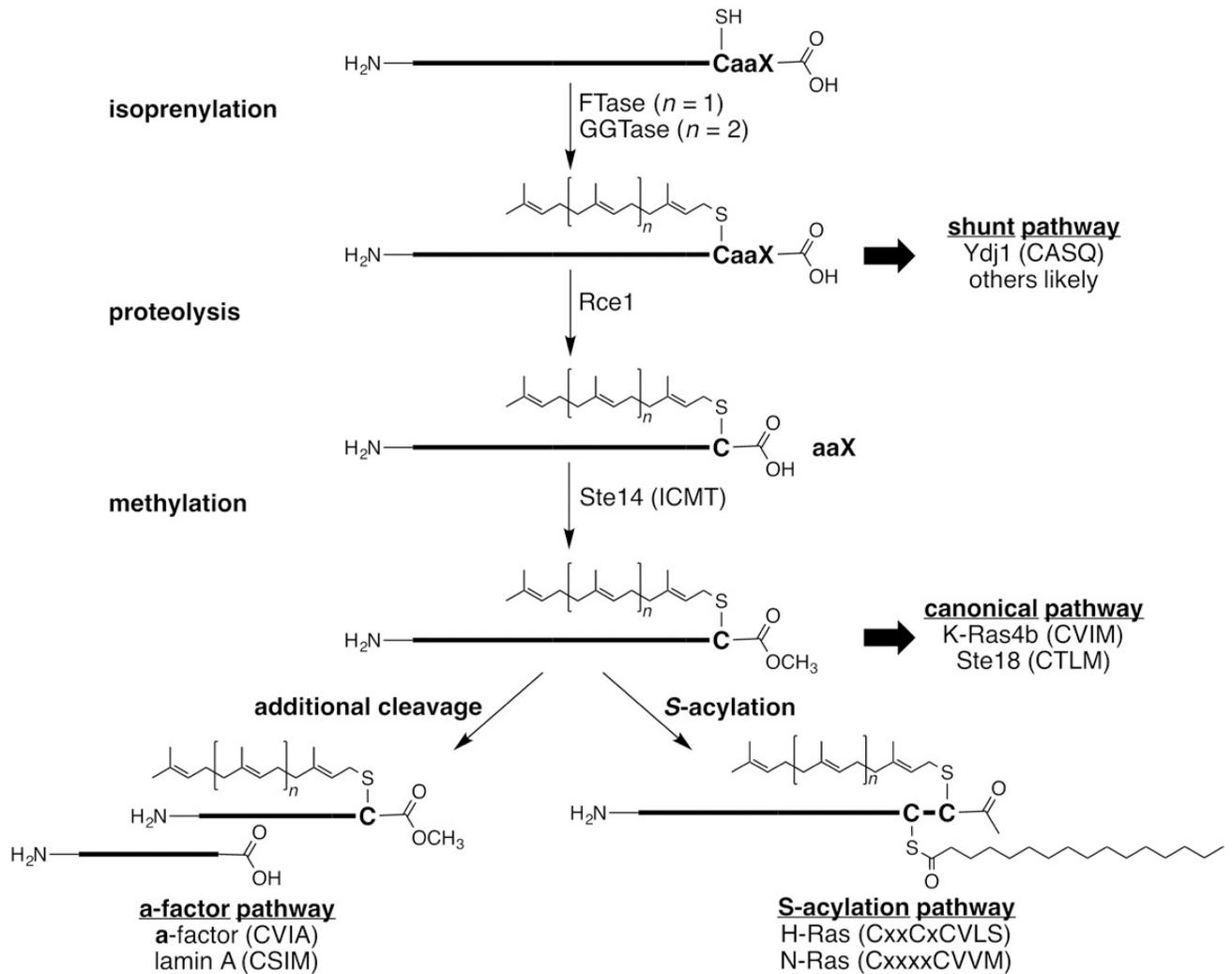
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**Figure 1.**

Overview of post-translational modifications associated with CaaX proteins. Both farnesyl (C15) and geranylgeranyl (C20) can be added to CaaX proteins. There are multiple classes of isoprenylated CaaX proteins: those with motifs that resist cleavage (shunt), those with motifs that are cleaved (canonical), and those additionally modified by cleavage (**a-factor**) or *S*-acylation. Motifs shown are for indicated yeast (Ydj1p, Ste18p, **a-factor**) or human proteins (all others).

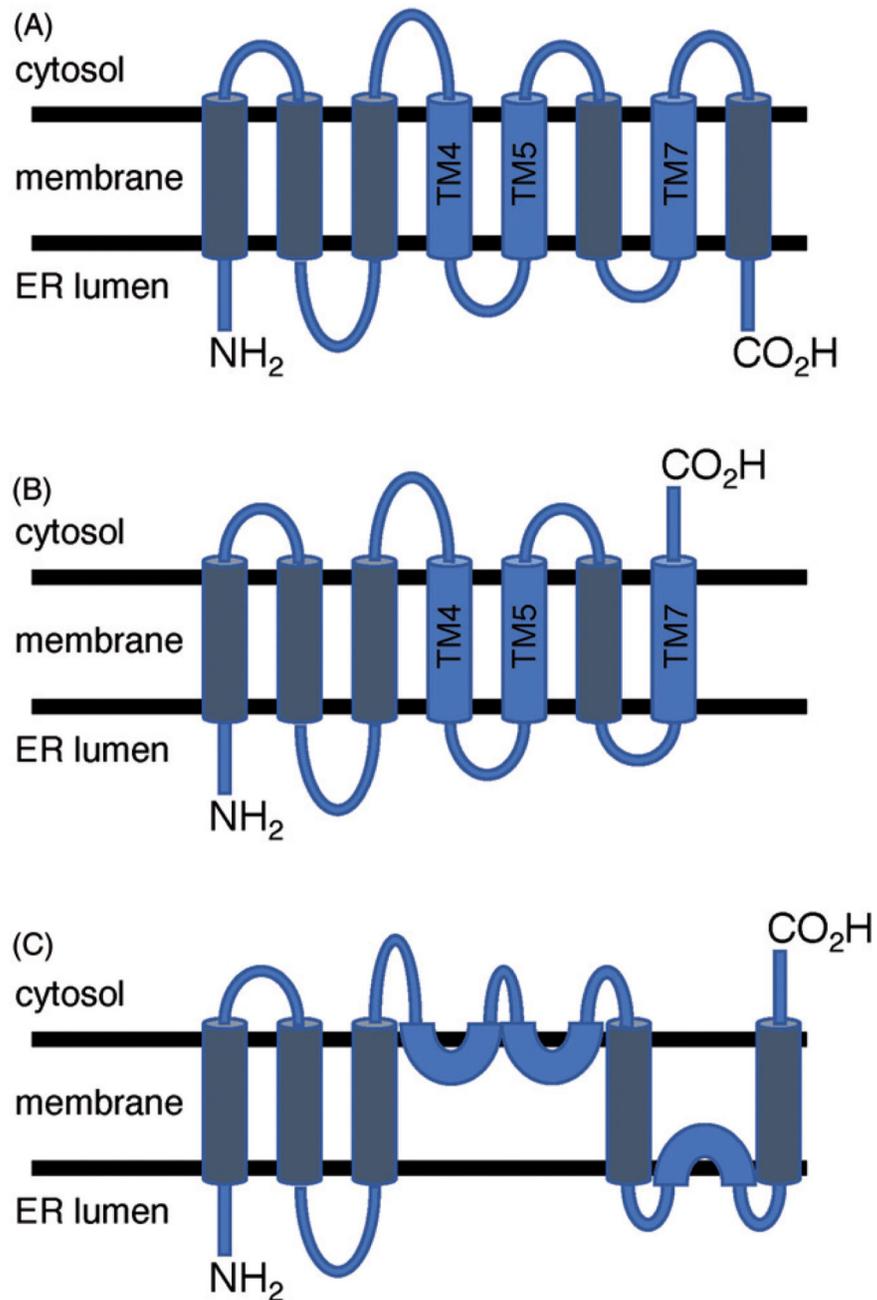


Figure 2. Postulated topology models of Rce1. (A) Topology with 8 transmembrane helices found in *MmRce1* crystal structure and predicted for *HsRce1*; (B) 7 transmembrane helices predicted for *ScRce1*; and (C) Proposed alternative topology for *ScRce1* based on substituted cysteine accessibility experiments. Conserved catalytic glutamate and histidine residues are located on blue-colored helices. (see color version of this figure at www.tandfonline.com/ibmg).

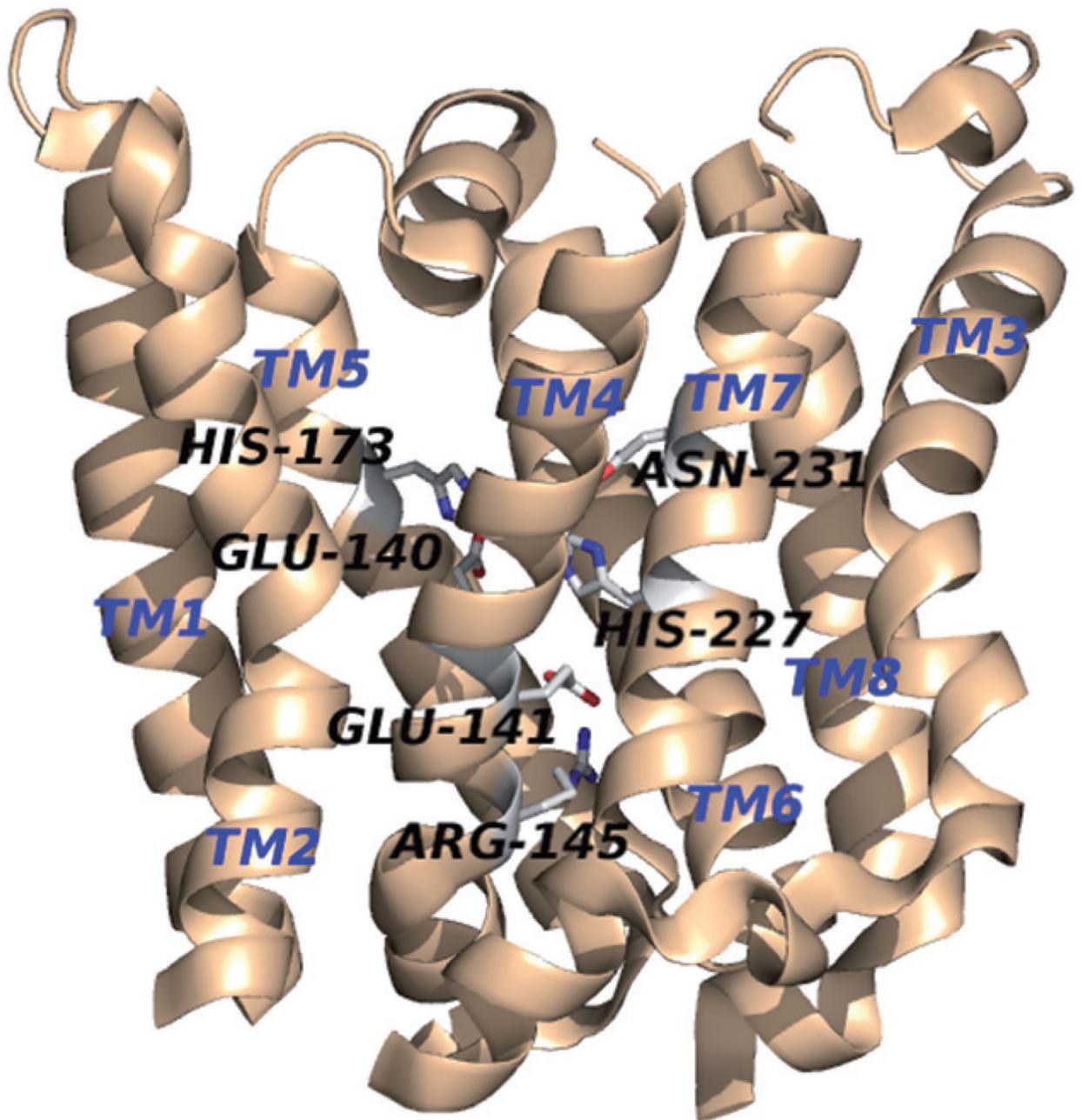


Figure 3.

Crystal structure of *MmRce1* (PDB: 4CAD). The protein structure is oriented with the cytosolic face at the top and that of the ER lumen on the bottom of the image. (see color version of this figure at www.tandfonline.com/ibmg).

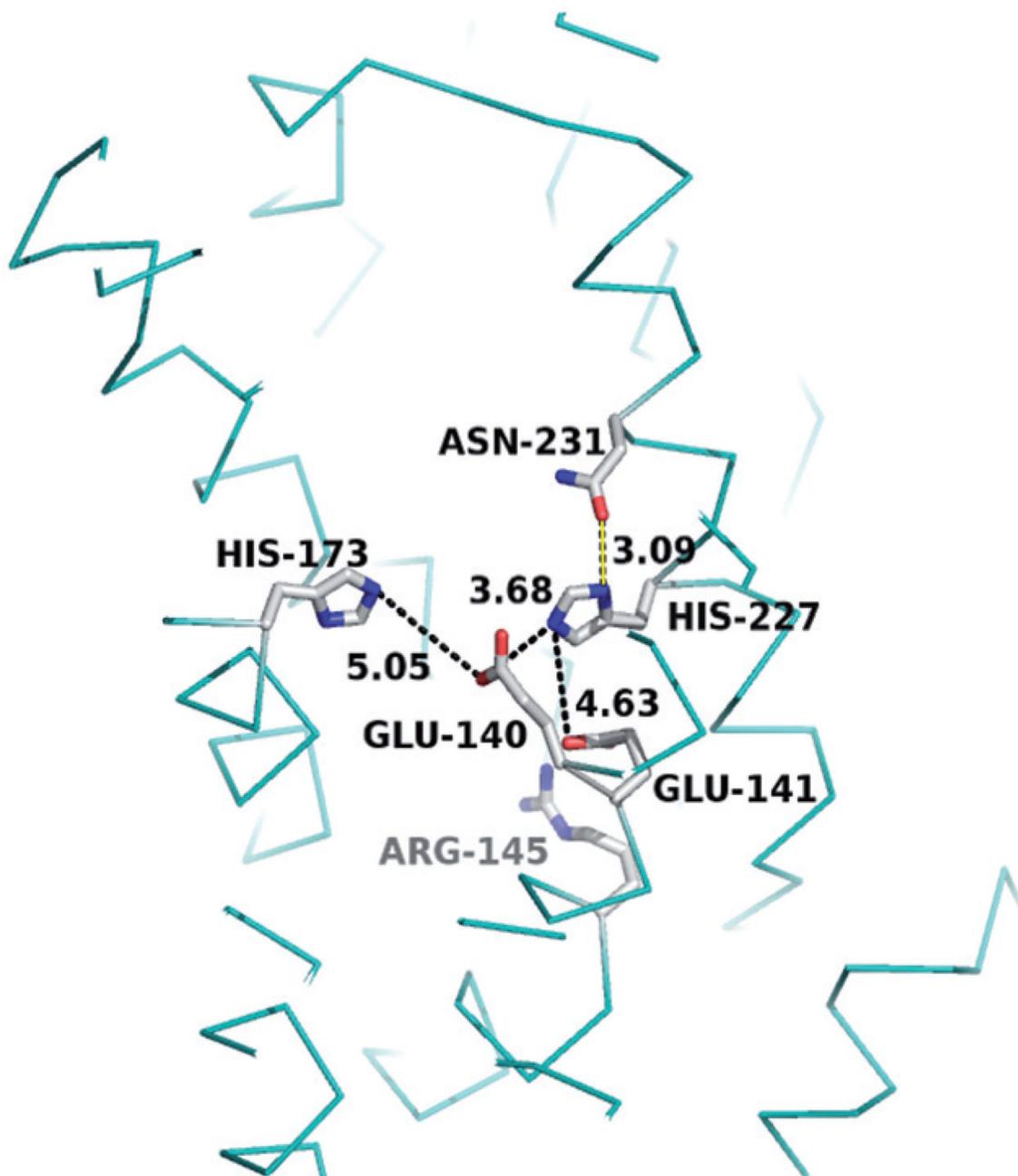


Figure 4. Crystal structure of the *MmRce1* catalytic site (PDB: 4CAD), showing the locations of the critical residues E140, E141, R145, H173, H227, and N231. Selected N–O distances between residues are given in Ångströms and marked by black dashed lines. The hydrogen bond between H227 and N231 reported by Manolaridis et al. is shown as a yellow line. (see color version of this figure at www.tandfonline.com/ibmg).

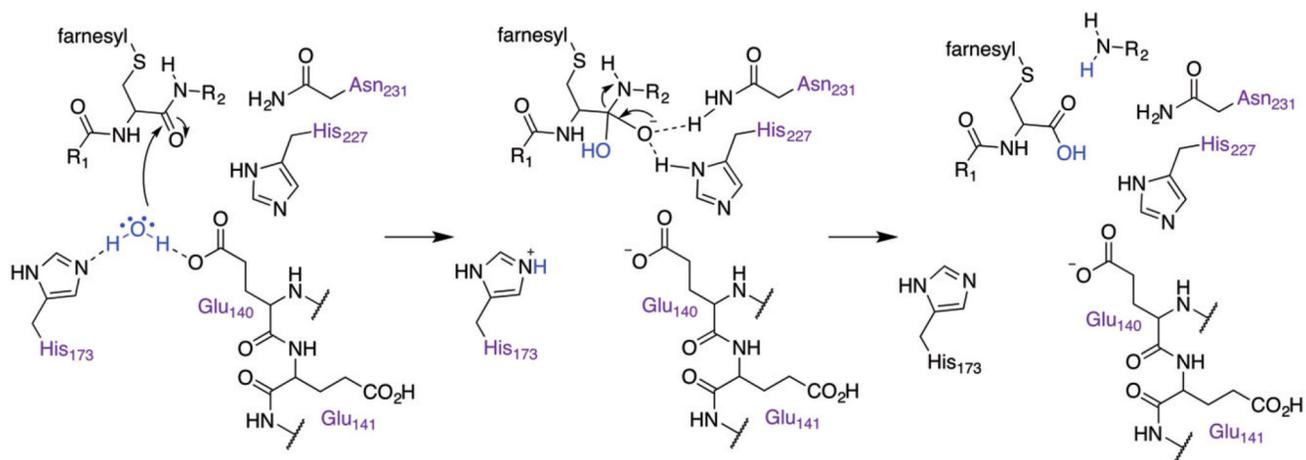


Figure 5.
Proposed basic catalytic mechanism of *MmRce1* proteolysis.

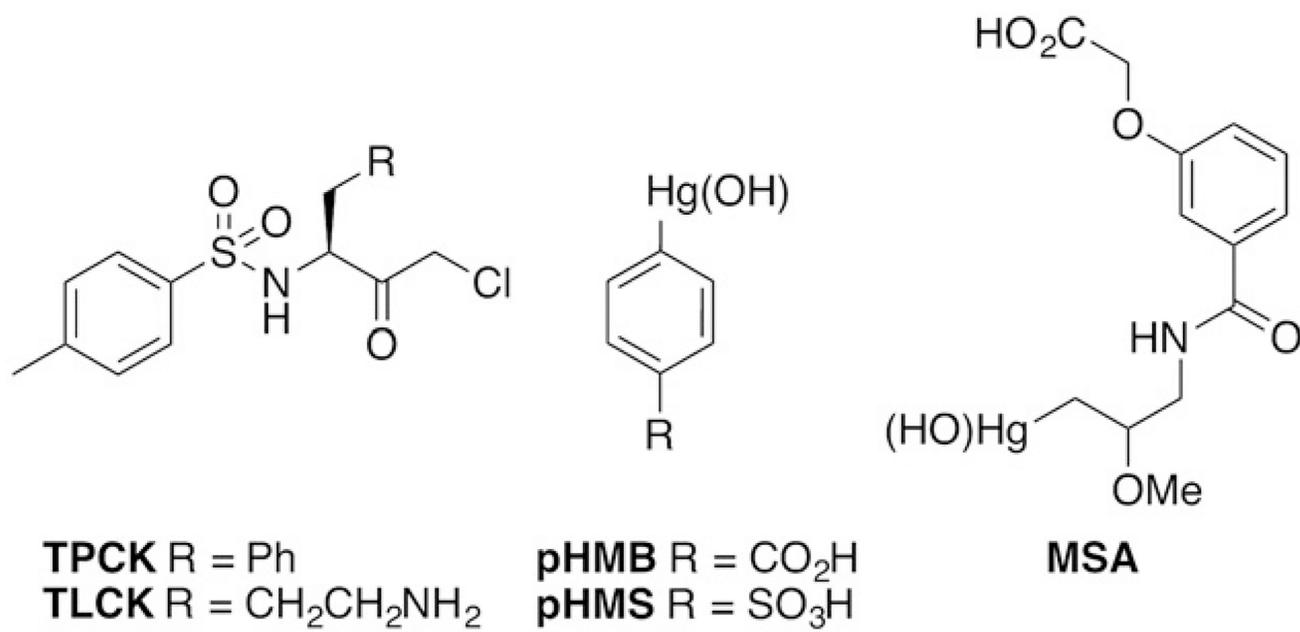
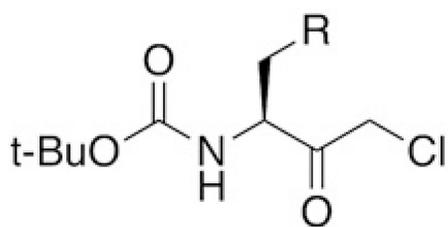
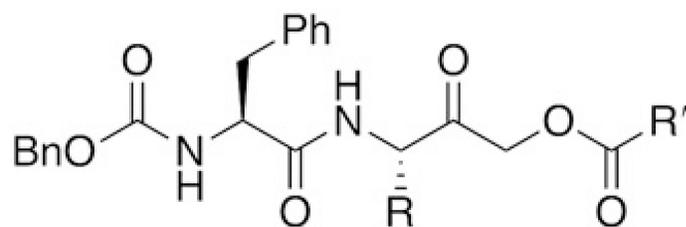


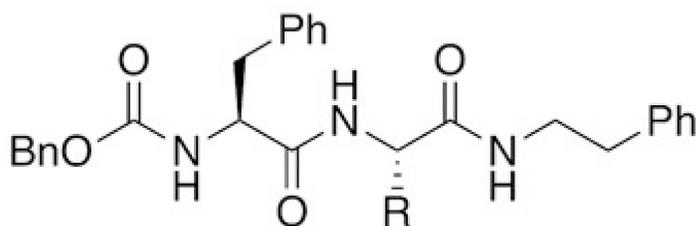
Figure 7.
 Structures of general protease inhibitors of Rce1.



BFCCMK R = S-farnesyl
UM96001 R = (CH₂)₁₂CH₃



dipeptidyl AOMKs
 R = (CH₂)₃NHC(NH)NH₂ or (CH₂)₄NH₂
 R' = aryl



"warhead-free" AOMK
 R = (CH₂)₃NHC(NH)NH₂

Figure 8.
 Structures of selected halo and acyloxymethyl ketone inhibitors of Rce1.

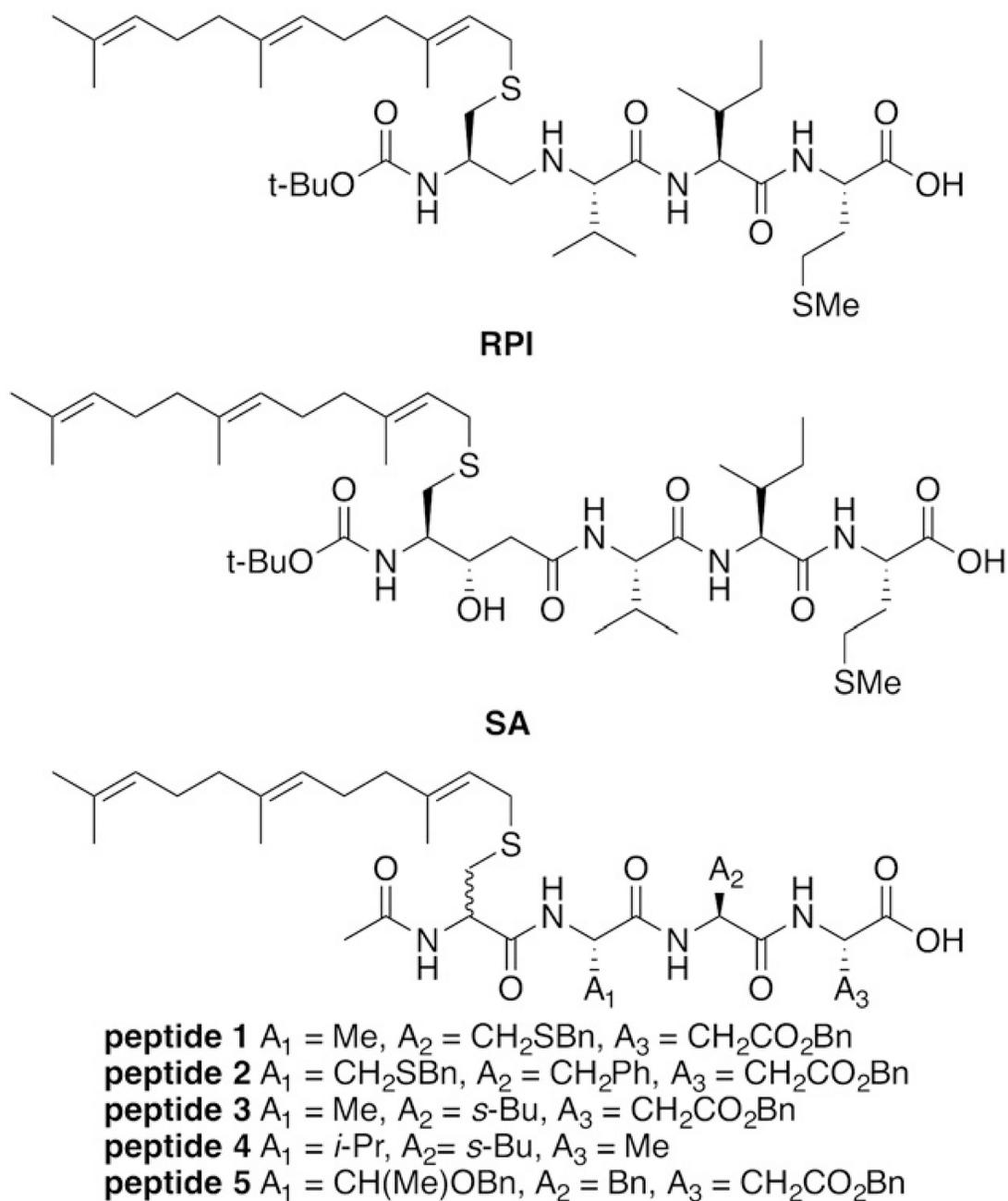


Figure 9.
Structures of RPI, SA, and selected RPI analogs.

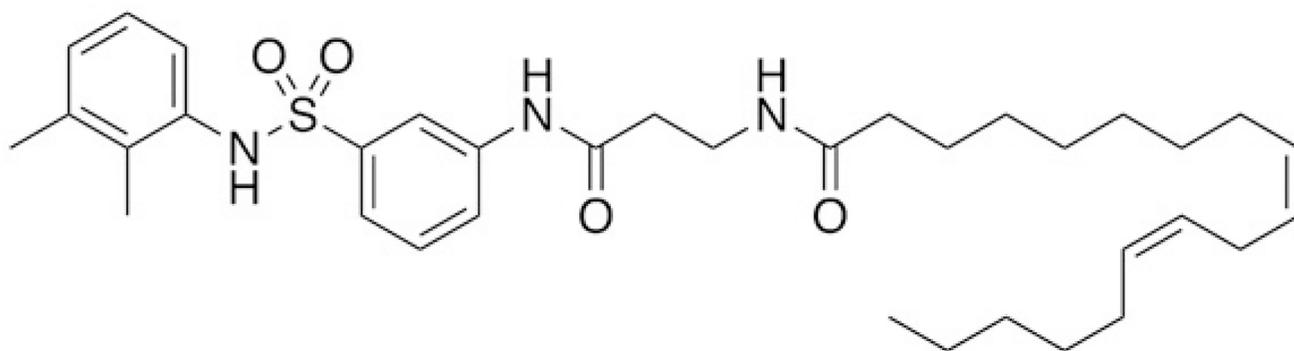
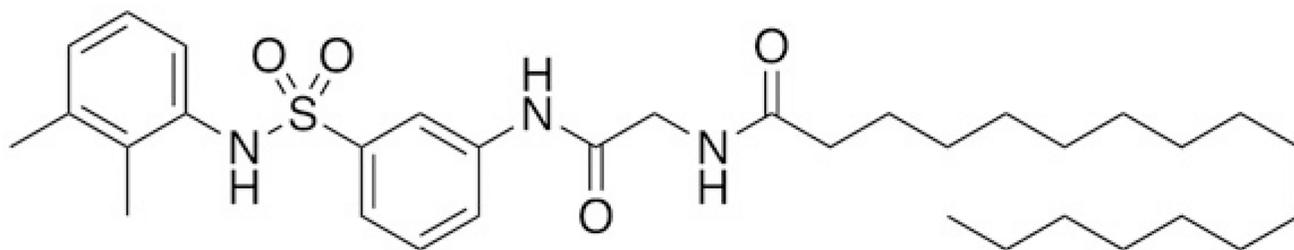
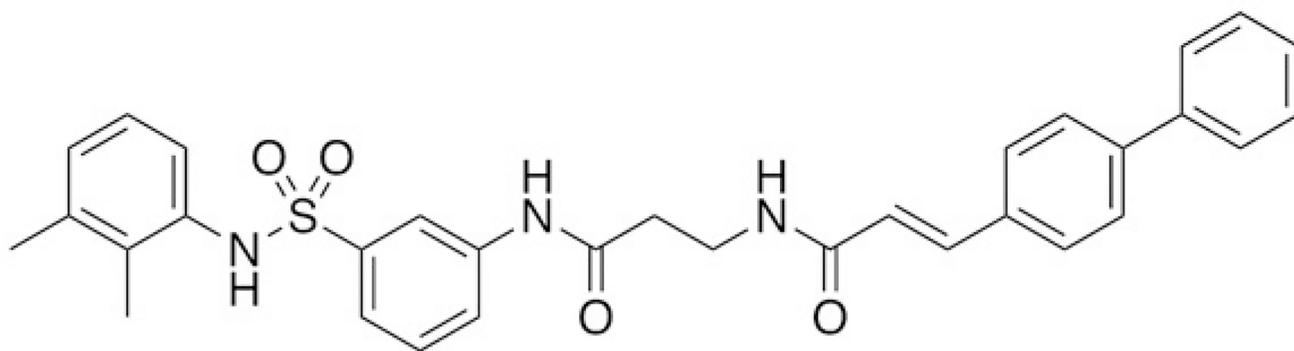
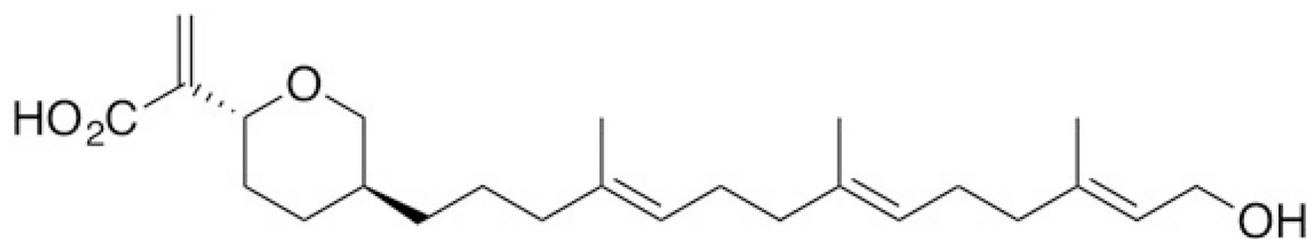
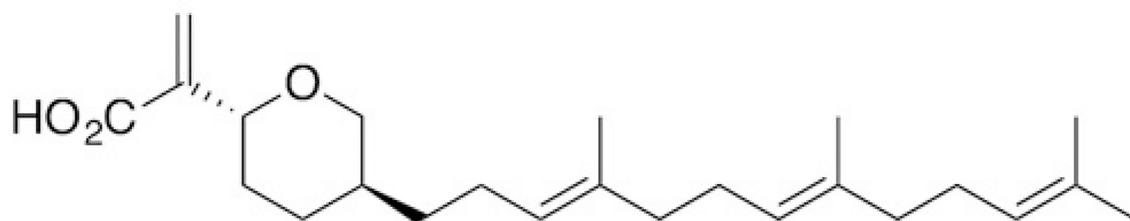
**NPNPI-A****NPNPI-B****NPNPI-C**

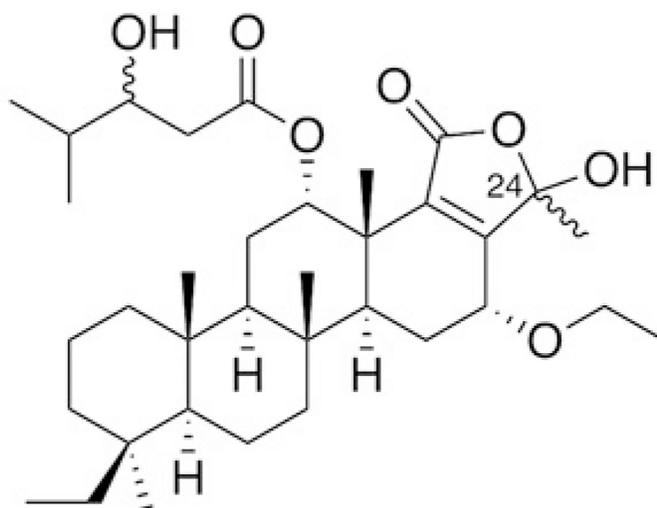
Figure 10.
Structures of peptidomimetic inhibitors.



barangcadoic acid A



rhopaloic acid A



20,24-bishomoscalarane ketals

Figure 11.
Structures of natural product inhibitors of Rce1.

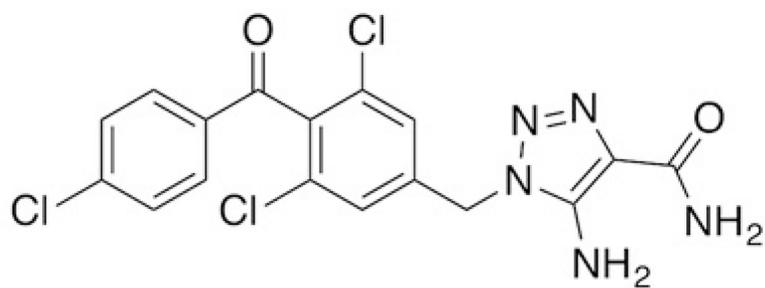
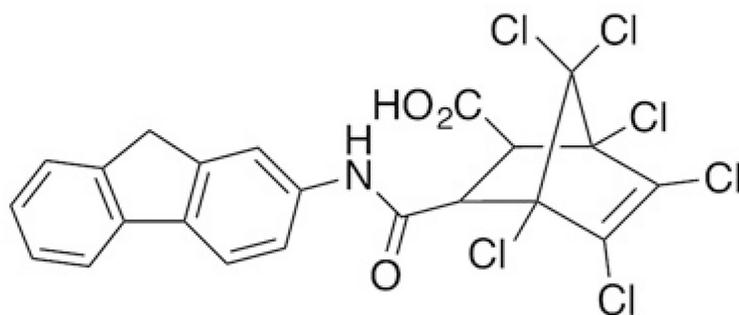
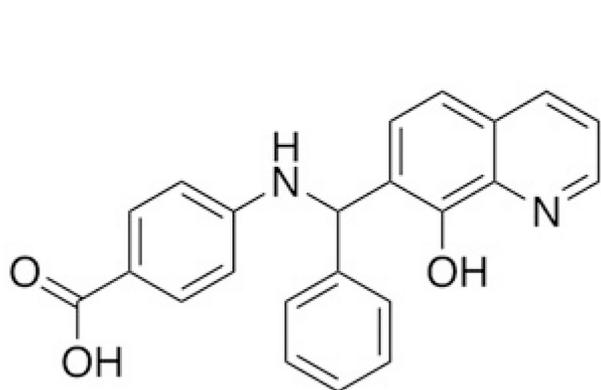
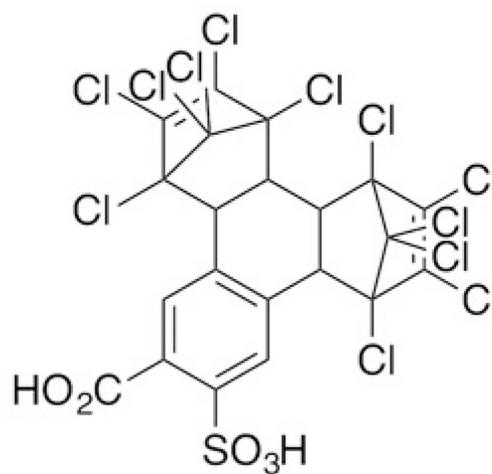
**NSC609974****NSC73101****NSC1011****NSC270718**

Figure 12.
Selected examples of Rce1 inhibitors identified in a medium throughput screen.

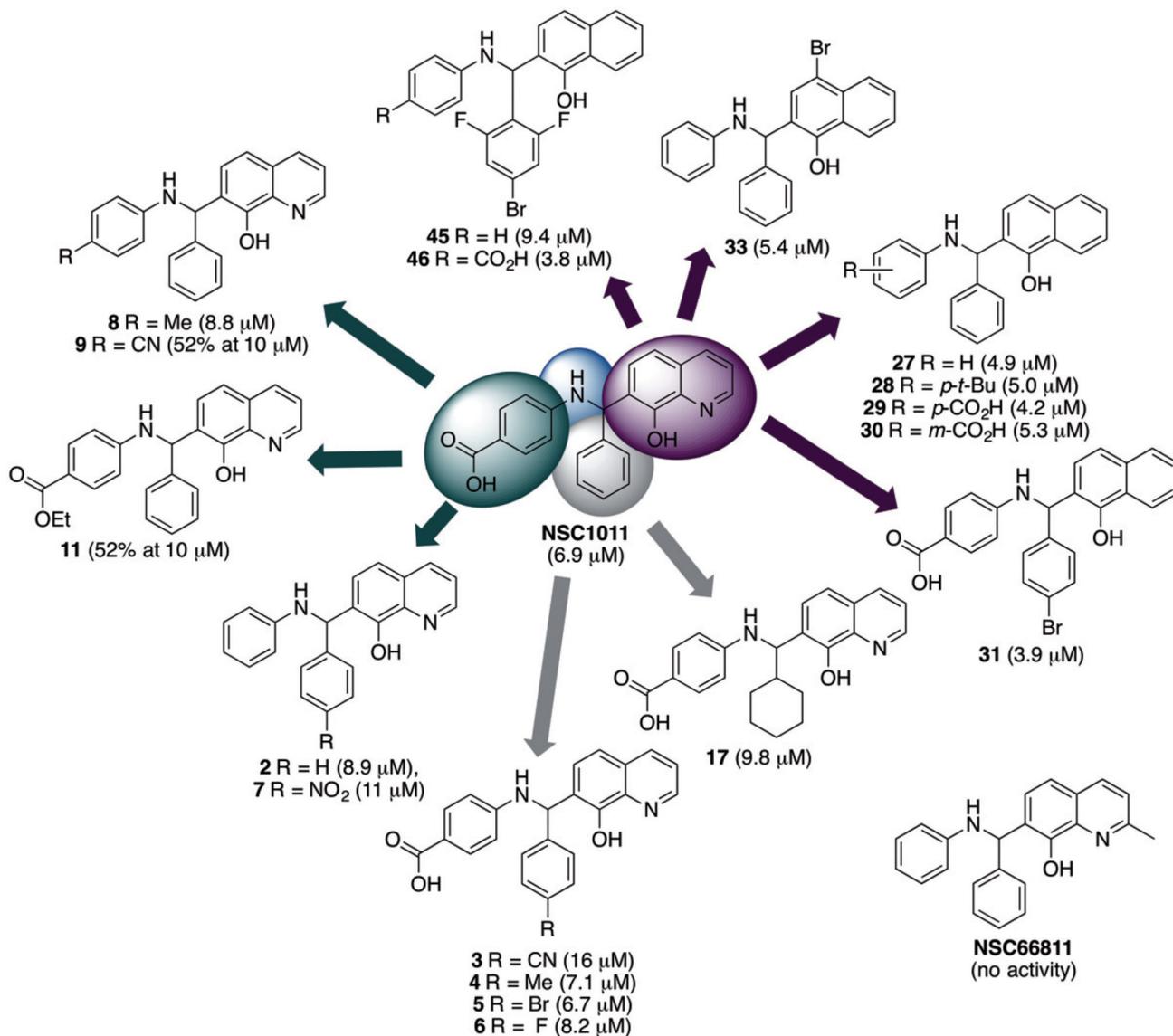


Figure 13.

Examples of NSC1011 derivatives that remained active against *HsRce1*. Numbers in parentheses are IC₅₀ values or the percent activity of Rce1 after 10 μM treatment as judged using the Rce1 IQF *in vitro* proteolysis assay (Mohammed et al. 2016). (see color version of this figure at www.tandfonline.com/ibmg).

Table 1Reporter substrates used to monitor Rce1 activity *in vivo* and *in vitro*.

Assay type	Reporter	Sequence ^a	Detection method
<i>In vivo</i>	GFP-Ras (H, N, K4a, K4b) (~45 kDa)	GFP-H-Ras-CVLS GFP-N-Ras-CVVM GFP-K-Ras4a-CIIM GFP-K-Ras4b-CVIM	Cell-based fluorescence
	GFP-Ras2p (~60kDa)	GFP-Ras2p-CIIS	Cell-based fluorescence
	a -factor precursor (36-mer)	Mature a -factor peptide-CVIA	Bioactivity
<i>In vitro</i>	a -factor CaaX (15-mer)	YIIKGVFWDPACVIA	Bioactivity, coupled
	a -factor (12-mer)	KGVFWDPACVIA	HPLC
	K-Ras4b (9-mer)	fluor-KSKTKCK ^Q IM	Fluorescence quenching assay
	a -factor CaaX (8-mer)	dansyl-WDPACVIA	HPLC
	a -factor CaaX (8-mer)	KWDPA CV[³ H]IA	HPLC, radiography
	Biopep 4-mer	bio-CVIA	Affinity purification, radiography

^aCysteine in the CaaX sequence is farnesylated. Abbreviations: fluor: *ortho*-aminobenzoic acid; K^QN⁶-(2,4-dinitrophenyl)-L-lysine; bio: 1-*N*-biotinyl-(13-*N*-suc-cinimidyl)-(S-farnesyl-1-cysteiny)-1-valinyl-1-isoleucinyl-[¹⁴C]-l-alanine)-4,7,10-trioxatridecanediamine.