Rhomboid proteases are increasingly being explored as potential drug targets, but their potent and specific inhibitors are not available, and strategies for inhibitor development are hampered by the lack of widely usable and easily modifiable in vitro activity assays. Here we address this bottleneck and report the development of new fluorogenic transmembrane peptide substrates, which are cleaved by several unrelated rhomboid proteases, can be used both in detergent micelles and in liposomes, and contain red-shifted fluorophores that are suitable for high-throughput screening of compound libraries. We show that nearly the entire transmembrane domain of the substrate is available for continuous activity assays that would capture all the native enzyme-substrate interactions, be applicable to both the detergent micelle system and liposomes, and would be easily adaptable for high-throughput screening of compound libraries. The fluorogenic substrates that we describe and systematically improved by targeted modification of the sequence of its P5 to P1 region. The fluorogenic substrates that we describe and their sequence variants should find wide use in the detection of activity and development of inhibitors of rhomboid proteases.

Rhomboid intramembrane proteases are evolutionarily widespread and regulate important biological processes including growth factor secretion (1, 2), mitochondrial dynamics (3), invasion of the malaria parasite (4), and membrane protein quality control (5). Rhomboid proteases are increasingly being explored as potential drug targets (6–9), but their selective and potent inhibitors are lacking (reviewed in Ref. 10). Rhomboid inhibitor discovery and development are complicated by the lack of widely usable and easily modifiable in vitro activity assays.

Rhomboid activity assays have traditionally relied on recombinant transmembrane protein substrates and gel-based readouts, but such assays are unsuitable for high-throughput screening. A fluorogenic substrate for the Providencia stuartii rhomboid protease AarA lacking most of the transmembrane domain of the parent substrate Gurken is cleaved very poorly by other rhomboids including the main model rhomboid protease GlpG of Escherichia coli (11). Other published variants of fluorogenic substrates can be used only in liposomes (12) or involve large fluorescent protein moieties making them dependent on expression in a biological system and photochemically less variable (13), which may be important for high-throughput screening of compound libraries where bright red-shifted fluorophores are preferred (14). Moreover, each of the described rhomboid substrates has been used only with one or two related rhomboid proteases, and a strategy to design widely usable or specific substrates has been lacking. Other types of activity assays employing MALDI mass spectrometry (15) and fluorescence polarization (16) have been reported, but MALDI is a low-throughput method that requires sophisticated instrumentation, and fluorescence polarization assays are based on competition of small molecular activity probes with inhibitors and are prone to detergent artifacts (16), making both of these methods unfit for routine kinetics measurements or high-throughput screening.

In view of these limitations, we have sought to develop a robust fluorogenic transmembrane peptide substrate platform for continuous activity assays that would capture all the native enzyme-substrate interactions, be applicable to both the detergent micelle system and liposomes, and would be easily adapt-
able to diverse rhomboid proteases. Because solid phase synthesis of transmembrane peptides and their purification are non-trivial, and their solution behavior often unpredictable, we place emphasis on choosing a robust system and characterizing it thoroughly, and present a generalizable framework for rhomboid substrate design.

**Results and Discussion**

LacYTM2 Is a Widely Accepted Rhomboid Substrate—To identify a substrate widely accepted by diverse rhomboid proteases, we have measured the efficiency of cleavage of four common model rhomboid substrate transmembrane domains (P. stuartii TatA, Drosophila melanogaster Gurken and Spitz, and E. coli LacYTM2) embedded in a chimeric construct by four unrelated rhomboid proteases (E. coli GlpG, Bacillus subtilis YqgP, P. stuartii AarA, and Bacteroides thetaiotaomicron rhomboid 3 (BtioR3)) (Fig. 1A). Comparison of the efficiencies of cleavage (molar catalytic activities) revealed that the substrate containing the second transmembrane (TM) helix of E. coli LacY protein (LacYTM2) (17) was the most “promiscuous” substrate.

Although it is well accepted that the region around the scissile bond, mainly P4 to P2', is key for the turnover efficiency of
rhomboid substrates (12, 18), the role of the TM domain of the substrate for recognition and catalysis by rhomboid is less well understood. We have thus next evaluated the importance of the transmembrane region of LacYTM2 for the recognition by E. coli GlpG, the main model rhomboid protease, by synthesizing a peptide covering the whole transmembrane region and adjacent juxtamembrane segments of LacYTM2, and a series of its C terminally truncated variants. The full-length LacYTM2 transmembrane peptide KSp31 was cleaved by GlpG efficiently and highly specifically at the expected Ser-Asp cleavage site (Fig. 2A). The kinetics of cleavage were monitored by capillary electrophoresis (Fig. 1C). The cleavage rate decreased significantly upon truncating the TM helix of LacYTM2 peptide by more than 5 amino acids from the C terminus (Fig. 1D), suggesting that most of the TM domain of the substrate is important for the interaction with and recognition by rhomboid. Thus, to develop a widely accepted fluorogenic substrate that would faithfully mimic all the relevant enzyme-substrate interactions including the intramembrane ones, we have used the full-length LacYTM2 transmembrane domain peptide KSp31 as a starting point.

**Fluorogenic Transmembrane Peptide Substrate Based on LacYTM2, Basic Properties**—To generate a fluorogenic variant of the LacYTM2 peptide, we have replaced the P5 and P4' positions in KSp31 by Glu-EDANS and Lys-DABCYL to yield KSp35 (Fig. 2A). Previously published mutagenic analyses show that these positions are not critical for recognition by rhomboid (18, 19), and they are sufficiently close for Förster resonance energy transfer (FRET) to occur. The KSp35 peptide was soluble up to 500 μM (Fig. 2B) in frequently used detergents at 16 mM decyl maltoside (DM), nonyl glucoside (NG), and dodecyl maltoside (DDM). At a total DDM concentration of 16 mM (0.82% (w/v)), the concentration of micelles is about 110 μM, suggesting a partitioning ratio of more than 1 molecule of the substrate per micelle. When DDM was kept at only 1 mM (0.05% (w/v)) total concentration, which yields about 6–10 μM micelles, the solubility of KSp35 became limited to about 100 μM (Fig. 2B), indicating that the upper limit of the partitioning ratio is about 10–20 molecules of KSp35 per DDM micelle. The solubility of KSp35 in the absence of detergent was negligible (not shown). Circular dichroism of KSp35 in 0.5% (w/v) DDM (Fig. 2C) showed a significant content of α-helical structure (61 ± 18%), which is consistent with the transmembrane character of the peptide and comparable with the helical content of the parent peptide KSp31 (54 ± 15%). Cleavage of KSp35 by GlpG occurred at the expected cleavage site (Fig. 2D), and was accompanied by an increase in fluorescence at 495 nm (Fig. 2E), demonstrating that FRET between the donor and acceptor is occurring in the uncleaved peptide. Collectively, the above results show that KSp35 is a realistic model reflecting all the

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**FIGURE 2.** Fluorogenic transmembrane peptide substrate based on LacYTM2. A, fluorogenic variant of the LacYTM2 transmembrane helix-derived peptide (KSp31) with the P5 and P4' positions replaced by Glu-EDANS and Lys-DABCYL, respectively, yielding fluorogenic substrate KSp35. B, solubility of KSp35 in 16 mM detergents DDM, DM, and nonyl glucoside (NG) and at 1 mM DDM. Note that the concentration of DDM micelles is about 100 μM at 16 mM DDM and about 10 μM at 1 mM DDM. The peptide was dissolved to the indicated concentration by dilution from a 10 mM stock solution in DMSO, and after a 2-h incubation at 37 °C the solution was centrifuged at 21,130 g for 20 min. The absorbance of the supernatant at 455 nm indicated the concentration of the chromophore in solution. C, circular dichroism spectra of LacYTM2-derived transmembrane peptide KSp31 and its fluorogenic variant KSp35 in detergent micelles. Peptides were reconstituted into 0.5% (w/v) DDM to 135 μM (KSp31) and 82 μM (KSp35) concentrations. The spectra show similarly significant helical content for both peptides. D, identification of the cleavage site in KSp35 by GlpG. Purified 95 μM KSp35 was incubated with 26 μM GlpG for 20 h and analyzed by MALDI. The red peak of the mass of 2993.7 corresponds well to the expected size of the C-terminal cleavage product of 2990.690. The second peak lower by 130 Da is probably a deletion product of chemical synthesis lacking a C-terminal lysine. This variant has proven difficult to purify away, but it is cleaved by GlpG and probably does not influence the kinetics properties of the substrate significantly (see Fig. 1D). E, excitation and emission spectra of KSp35 and their change upon cleavage by rhomboid GlpG measured in detergent micelles. The spectra of 10 μM KSp35 substrate in reaction buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% (w/v) DDM, 10% (v/v) DMSO) were measured at 37 °C. Excitation wavelengths ranged from 235 to 435 nm with a 10-nm increment and the emission was measured at 493 nm. The emission wavelengths ranged from 365 to 595 nm with a 10-nm increment and excitation at 335 nm.
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important interactions between a rhomboid protease and its transmembrane substrate.

Kinetic Characterization of the LacYTM2-based Substrate KSp35 in Detergent Micelle System—In the detergent-solubilized state, most commonly used to study the biochemistry of intramembrane proteolysis, the reaction catalyzed by rhomboid protease occurs in detergent micelles due to the hydrophobicity of both enzyme and substrate. The system is thus microheterogeneous, the effective concentrations of the reactants depend on the volume of the micellar milieu and on the partitioning of reaction components between free solution and the micelles. To characterize the kinetic behavior of the new fluorogenic transmembrane substrates in light of these features of the micellar system, steady-state kinetics was measured with 10 μM substrate, 0.4 μM enzyme, and 0.05% (w/v) DDM, always keeping the concentrations of two components constant and varying the third one around the stated values. At 0.05% (w/v) DDM, the concentration of detergent monomers is 980 μM and micelle concentration about 6–10 μM, calculated assuming critical micellar concentration (CMC) of 0.17 mM (20) and dehydrated state, most commonly used to study the biochemistry of intramembrane proteolysis, the reaction catalyzed by rhomboid protease occurs in detergent micelles due to the hydrophobicity of both enzyme and substrate. The system is thus microheterogeneous, the effective concentrations of the reactants depend on the volume of the micellar milieu and on the partitioning of reaction components between free solution and the micelles. To characterize the kinetic behavior of the new fluorogenic transmembrane substrates in light of these features of the micellar system, steady-state kinetics was measured with 10 μM substrate, 0.4 μM enzyme, and 0.05% (w/v) DDM, always keeping the concentrations of two components constant and varying the third one around the stated values. At 0.05% (w/v) DDM, the concentration of detergent monomers is 980 μM and micelle concentration about 6–10 μM, calculated assuming critical micellar concentration (CMC) of 0.17 mM (20) and aggregation number between 78 and 149 (20). The molar ratio of enzyme:substrate:micelles is thus 4:100:60–100. In these conditions, assuming that all the reaction partners are evenly distributed among micelles, the average number of substrate molecules per micelle is about 1.5, and only up to 4% of micelles carry an enzyme molecule (micelles containing more than one enzyme molecule are strongly improbable).

The cleavage reactions were started by either mixing two preheated solutions containing substrate or enzyme preincubated with detergent, or adding the DMSO-dissolved substrate into the rest of the preheated reaction mixture. In either case, progress curves are linear from the beginning, which implies that the redistribution of the adsorbed molecules among the micelles is significantly faster than substrate cleavage itself. In accordance with this, the reaction rate is proportional to enzyme concentration within the 0–0.6 μM range (Fig. 3A). Within this concentration range, few enzyme molecules are randomly distributed among many more micelles, providing in principle equal conditions for each enzyme molecule. A similar principle can also explain the observation that the dependence of the reaction rate on substrate concentration is linear in the 0–4 μM range (Fig. 3B). At the upper limit of 4 μM substrate, all micelles can be populated by one (or less likely more) substrate molecule, the linear dependence, furthermore, suggests that this substrate concentration is still below the apparent Michaelis constant of this process.

An important phenomenon is observed when the dependence of the initial rate on detergent concentration is measured. At concentrations above the CMC, the reaction rate rapidly decreases as DDM concentration grows (Fig. 3C), without an obvious impact on the secondary structure content of GlpG (Fig. 3D), suggesting that the effect is caused primarily by the increase in the volume of the micellar phase and consequent decrease of the effective concentrations of both substrate and enzyme. Indeed, mathematical consideration suggests that when substrate and enzyme concentrations are significantly lower than the concentration of micelles (i.e. at high DDM concentrations), the probability of location of a substrate molecule on the same micelle as the enzyme molecule is inversely proportional to the concentration of DDM. Under these conditions, the fraction of substrate-occupied micelles, f_{SM}, is equal to the ratio of the numbers of substrate molecules, n(S), and micelles n(M).

\[ f_{SM} = \frac{n(S)}{n(M)} \]  

(1)

The mean number of micelles occupied by both the enzyme and substrate molecules, n(ESM), is then given by this fraction multiplied by the number of enzyme molecules n(E).

\[ n(ESM) = f_{SM} \times n(E) = \frac{n(S)}{n(M)} \times n(E) \]  

(2)

Hence, when the DDM concentration is increased at constant n(S) and n(E), then n(ESM) reflecting the reaction rate decreases in accord with the growing value of n(M). This causes the proportional decrease of the reaction rate (in other words, the reaction rate is proportional to [DDM]^{-1}). To inspect whether this model is correct, one can conveniently determine the power of the measured rate dependence on DDM concentration by taking a logarithm of the data from Fig. 3C (log a^{[DDM]} = n \times \log d). The logarithmic plot (Fig. 3C, open circles, right and upper axes) can be satisfactorily (R^2 = 0.9974) fitted by a second-order polynomial, yielding equation: y = −0.1436x^2 − 0.3906x + 2.8852, whose derivative y’ = −0.2872x − 0.3906 indicates the power of DDM concentration on which the reaction rate depends. This analysis shows that for high DDM concentrations the derivative indeed tends to −1 (for x = 2, y’ = −0.965; thus rate ∼[DDM]^{−1}), which is in accordance with the above assumption, whereas for the lower end of DDM concentrations the absolute value of the power decreases (for x = 0, y’ = −0.3906; thus rate ∼[DDM]^{−0.4}). This is consistent with a model that upon decreasing the detergent concentration (while still being above the CMC), the density of the adsorbed molecules in the micellar phase increases, whereas total concentration of micelles decreases, which leads to less frequent collisions between them and thus less effective redistribution of the adsorbed molecules among the micelles. Possibly, the redistribution efficiency might also be insufficient because of the higher reaction rate caused by the higher reactant concentrations.

Although the reaction kinetics of intramembrane proteases in liposomes has been described in terms of interfacial kinetics (12, 21), that is, expressing the kinetic constants in relationship to the volume or molar fraction of the lipidic phase, (22, 23), the kinetic effects related to the reaction occurring in detergent micelles have surprisingly not yet been considered in enzyme kinetics studies on rhomboid proteases (12, 13) nor other intramembrane proteases, yet they are evidently important for the interpretation of kinetics measurements. Our data show that for reliable and meaningful measurement of apparent Michaelis-Menten kinetics parameters, the micelle concentration must not be limiting the solubility of the substrate, and the detergent concentration must be kept constant. The latter point also means that having a stock solution of the substrate dissolved in detergent (at a higher concentration than intended in the reaction mixture, which frequently can occur during purification and concentration) may lead to underestimation of reaction rates at high substrate concentrations due to a possibly
significant increase of detergent concentration in the final reaction mixture, as shown in Fig. 3C. This could result in pseudo-Michaelis kinetics and yield falsely low $K_m$ values. Practical implications are that 1) exact detergent concentrations must be known in any kinetics measurements, and 2) it is advantageous to have the substrate stock solution dissolved in a detergent-free medium or at a detergent concentration lower or equal to that used in the final assay buffer. The transmembrane substrates presented in this article, generated by chemical synthesis, are in principle avoiding this problem, because their stock solutions are detergent-free dissolved in anhydrous dimethyl sulfoxide. Alternatively, they can be reconstituted into a detergent of choice via disaggregation in hexafluoroisopropanol, as described by Deber et al. (24).
The pH dependence of cleavage rate of the unmodified LacYTM2 transmembrane segment in the context of an MBP-thioredoxin fusion protein shows a relatively broad maximum around pH 9, with substantial activity of GlpG between pH 6 and 11 and negligible activity below pH 4 and at pH 12 (Fig. 3E), which is largely in agreement with previous studies (12, 13). The dependence of the cleavage rate of KSp35 on pH also shows that GlpG is completely inactive at pH values below and up to 4, but the initial reaction rate of KSp35 cleavage then appears to grow up to pH 12 (Fig. 3F). This effect cannot be ascribed to the pH-dependent change of EDANS fluorescence (data not shown), and could possibly be due to effects of pH on the conformational dynamics of KSp35. However, this is not a concern because in most cases measurements are performed at a physiologically relevant pH near neutral. The apparent catalytic efficiency $k_{cat}/K_m$ of GlpG against KSp35 measured at pH 7.4 and 0.05%(w/v) DDM is $(2.0 \pm 0.5) \times 10^{-3} \text{ min}^{-1} \text{ M}^{-1}$, which is comparable with the values reported for the TatA substrate by Dickey et al. (12) and Arutyunova et al. (13) obtained in similar conditions. Importantly, the LacYTM2-derived fluorogenic peptide substrate KSp35 is cleaved efficiently by unrelated recombinantly purified bacterial rhomboids GlpG, AarA, and BtoR3, and modestly by YqgP (Fig. 3G), which demonstrates its wide usability, surpassing any other currently available rhomboid substrates.

Use of the Transmembrane Peptide Substrate in Liposomes—Because the natural environment of rhomboid proteases is the lipid membrane, we next tested whether the fluorogenic peptide substrate KSp35 can also be used in liposomes. We co-reconstituted KSp35 with GlpG or its inactive mutant S201A/H254A at pH 4 into large unilamellar vesicles (LUVs) formed from E. coli polar lipid extract and confirmed the composition of the resulting proteoliposomes by SDS-PAGE (Fig. 4A). Negative stain transmission electron microscopy showed that both empty LUVs and proteoliposomes containing KSp35 in the presence or absence of GlpG or its inactive mutant S201A/H254A had similar morphology and size distribution both at pH 7.4 and 4 (Fig. 4B). The CD spectrum of LUV-reconstituted KSp35 showed helicity of 50 ± 14% (Fig. 4C), which is consistent with its transmembrane helix prediction. GlpG is inactive at pH 4 (Fig. 3, E and F), and, consistently, fluorescence of proteoliposomes containing KSp35 and GlpG at pH 4 was at a constant background level (Fig. 4E). Upon neutralization to pH 7.4, time-dependent increase of fluorescence at 495 nm was observed in the presence of wild type GlpG but not in the presence of its active-site mutant S201A/H254A (Fig. 4D). These results collectively demonstrate that the LacYTM2-based fluorogenic transmembrane substrate KSp35 is widely usable both in detergent micelles or liposomes and with diverse rhomboid proteases.

A Red-shifted Variant of the Fluorogenic Transmembrane Substrate for Rhomboids—Large compound libraries for high-throughput screening can often contain compounds that absorb in the UV region (14), and fluorogenic substrates operating at red-shifted wavelengths are less affected by such compound interference. Because EDANS is excited in the UV region, and is thus prone to interference in library screening, we have modified the LacYTM2 peptide backbone by instead attaching the red-shifted TAMRA fluorophore to a Lys introduced into the P5 position and a compatible dark quencher QXL610 to a Cys introduced into the P4’ position (Fig. 5A) to yield KSp76. This red-shifted fluorogenic substrate is cleaved by several bacterial rhomboid proteases with efficiencies similar to its UV variant KSp35. The apparent catalytic efficiency $k_{cat}/K_m$ of GlpG cleaving KSp76 is $(1.6 \pm 0.5) \times 10^{-3} \text{ min}^{-1}$.
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A modification of Lys in the P5 position of KSp31 by the red-shifted TAMRA fluorophore and P4 ‘Cys by a dark quencher QXL610 yields highly fluorogenic substrate KSp76 that is efficiently cleaved by rhomboid proteases GlpG, AarA, YqgP, and BtioR3 at identical concentrations to those used in Fig. 3G. Excitation wavelength was 553 nm, and emission was followed at 583 nm. The dose-response curves of the substrates to improve their kinetic properties and adapt them for screening assays. Furthermore, we provide a strategy how to obtain accurate kinetic data. We expect that the substrates we describe and sequence variants thereof will enable facile screening of inhibitors of rhomboid proteases.

In summary, we report novel sensitive versatile fluorogenic transmembrane peptide substrates for rhomboid intramembrane proteases that are usable both in detergent micelles and liposomes, are cleaved by diverse rhomboid proteases, and contain a red-shifted fluorophore suitable for high-throughput screening assays. Furthermore, we provide a strategy how to adapt these substrates to individual rhomboid proteases by modifying their P5 to P1 residues, and we demonstrate that controlling the detergent concentration is important for obtaining accurate kinetic data. We expect that the substrates we describe and sequence variants thereof will enable facile detection of activity and development of inhibitors of rhomboid proteases.

Experimental Procedures

General Biochemicals—Lipids were from Avanti Polar Lipids, detergents from Anatrace, buffers and other biochemicals were from Sigma or other suppliers as specified below.

DNA Constructs and Cloning—The expression constructs for rhomboid proteases GlpG, YqgP, and AarA and chimeric MBP-TMD-Trx substrate constructs where TMD = LacYTM2, Gur-
Fluorogenic Substrates for Rhomboid Proteases

FIGURE 6. The effect of non-prime side substitutions on the catalytic parameters and selectivity of rhomboid substrates. A, preferred amino acids in the P5 to P1 positions of the LacYTM2 transmembrane substrate improve its cleavage by GlpG. The LacYTM2 embedded in the MBP-thioredoxin chimeras (18) was point-mutated in the P5 to P1 positions according to the sequence preferences of E. coli GlpG (19). The recombinant substrates were expressed in E. coli ΔglpG, purified, and molar catalytic activity of GlpG in cleaving each of the substrates was determined using gel-based assay (see “Experimental Procedures” for details). The concentration of substrate was always 1.47 μM, concentration of DDM was 0.5% (w/v), the concentration of GlpG was 0.8 μM for wild type substrate (HISKS), and for the RISKs, HISKs, and HISKS mutants the concentration was 0.08 μM for the HISKa mutant and 0.016 μM for the HIRKS and RVHRA variants (to ensure reliable measurement of the initial reaction rate). Representative values from one of three independent experiments are shown. B, the effects of the preferred amino acids in the P5 to P1 region of LacYTM2 on the steady-state level of cleavage by GlpG in biological membranes in vivo. Plasmids encoding individual mutant versions of the chimeric mutant LacYTM2 substrates described above were transformed into E. coli MC4100 expressing endogenous GlpG, and 2 h after induction of expression of the substrates, the cell lysates were analyzed by immunoblotting using antibody against His tag, located at the C terminus of the constructs. Detection by near-infrared laser scanning, exhibiting linearity over 6 orders of magnitude, enabled reliable quantitation. Integration of product and substrate band intensities yielded steady-state substrate conversion values that are listed below the image. A representative experiment is displayed. C, apparent kinetic parameters of fluorogenic rhomboid substrates derived from LacYTM2. Initial reaction rates at very low substrate concentrations were used to calculate catalytic efficiency values (kcat/Km) of substrates KSp35, KSp64, and KSp76 cleaved by GlpG at 0.5% (w/v) DDM. The reaction buffer was 20 mM HEPES, pH 7.4, 150 mM NaCl, 10% (v/v) DMSO, enzyme concentration was 0.4 μM, and substrate concentration ranged from 0.5 to 20 μM. Note that a mere optimization of the P5 to P1 region of the substrate increases the catalytic efficiency (kcat/Km) of its cleavage by GlpG by 23-fold. D, influence of the optimization of the P5 to P1 region on the selectivity of a transmembrane substrate for rhomboids. KSp76 underwent cleavage by rhomboid proteases GlpG, AarA, YqgP, and BtioR3 at the same concentrations as described in the legends to Figs. 3G and 5A. Note that optimization of the P5 to P1 region of the substrate increases the selectivity for GlpG dramatically.

Protein Expression and Purification—Bacterial rhomboid proteases AarA, GlpG, BtioR3, and YqgP and the active site mutant GlpG.S201A were overexpressed in E. coli C41(DE3) (30) as full-length, C terminally His-tagged proteins from a modified pET25b+ vector (27). The cultures were grown at 37 °C in LB medium to A600 of 0.4 and induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside. The expression was continued overnight at 20 °C. Cells were harvested, resuspended in buffer A (25 mM HEPES, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 1 mM PMSF), and lysed by 2 to 3 passes through Avestin EmulsiFlex-C3. Cell debris was removed by a low-speed centrifugation. Cellular membranes were isolated by a 2-h centrifugation at 100,000 × g and were solubilized in 1.5% (w/v) DDM (solubilization grade, Anatrace) in Buffer B (25 mM HEPES, pH 7.4, 300 mM NaCl, 10% (v/v) glycerol, 10 mM imidazole, EDTA-free Complete Protease Inhibitor mixture (Roche Applied Science)) at room temperature for 1 h. Solubilized proteins were isolated by centrifugation at 100,000 × g for 30 min and loaded onto nickel-nitrilotriacetic acid HiTrap IMAC HP 1-mL columns (GE Healthcare). Nonspecifically bound proteins were verified by DNA sequencing.

Chemical Synthesis—All reagents were acquired from commercial sources and used without purification. Protected amino acids and amino acid derivatives were purchased from Iris Biotech (Marktredwitz, Germany). Triphenyl phosphane was from AnaSpec (Fremont, CA), and 1-N-(9-fluorenylethoxy) carbonyl (Fmoc)-Glu(EDANS)-OH from Merck KGaA (Darmstadt, Germany). The detailed synthetic procedures, analytical methods, and compound characterization data are included in the supporting information.
MalDI-TOF mass spectrometry on an UltrafleXtreme™ interest and the internal standard (tyramine) (32). 

rected (migration time normalized) peak areas of peptides of 
with fresh BGE after each run. All analyses were performed in 
capillary injection end). The electrode vessels were replenished 
by guest on April 25, 2019http://www.jbc.org/Downloaded from

buffer exchanged into 25 mM HEPES, pH 7.4, 150 mM NaCl, 
10% (v/v) glycerol, and 0.05% (w/v) DDM on a HiPrep 26/10 
desalting column (GE Healthcare). If needed, proteins were 
concentrated using Vivaspin ultrafiltration spin cells with 
30-kDa MWCO. Protein concentration was determined from 
absorbance at 280 nm, and the final concentration of DDM was 
determined as described (31).

Capillary Electrophoresis (CE)—Analyses of standard pep-
tides and enzymatically cleaved peptide substrates were per-
formed on an Agilent CE 7100 instrument (Agilent, Wald-
bronn, Germany) equipped with photodiode array UV-visible 
detector operating in the 190–600 nm range. Electrophero-
grams were acquired at 192, 205, and 214 nm and absorbance 
data at 192 nm were selected for quantitative evaluation due to 
the highest signal to noise ratio. CE analyses were carried out in 
a bare fused silica capillary with polyimide outer coating (inter-
nal diameter 50 μm, outer diameter 375 μm, effective length to 
the detector 40 cm, total length 48.5 cm, supplied by Polymicro 
Technologies, Phoenix, AZ). Peptides were analyzed as cations 
in acidic background electrolyte (BGE) composed of 100 mM 
H₃PO₄, 69 mM Tris, pH 2.5. For highly hydrophobic peptides, 
this BGE was modified by the addition of 0.05% (w/v) DDM.

The temperature of the air-cooled capillary was set to 20 °C 
and the sample carousel was kept at the same temperature using a 
circulating water bath. Prior to each CE run, the capillary was 
successively washed with 100 mM sodium dodecyl sulfate, eth-
anol, 1 M NaOH, water, 1 M HCl, and the BGE, to remove any 
possible carryover of hydrophobic peptides and detergents 
from the previous run. All washes were done at 8 bar pressure 
for 30 s. Peptide standards used for identification of cleavage 
products were solubilized in DMSO at 1 mM concentration and 
mixed with 50 mM HEPES buffer containing 0.05% (w/v) DDM, 
resulting in 50 μM peptide concentration.

The enzymatic cleavage reactions were carried out in 20 mM 
HEPES, pH 7.4, with 0.05% (w/v) DDM and 10% (v/v) DMSO, 
with 250 μM peptide substrate and 2.6 μM full-length GlpG at 
37 °C. To measure the initial reaction rates, fractions were col-
lected every 15 min for up to 2 h and the reaction was termi-
nated by the addition of 10 mM HCl. Samples for CE were 
prepared by mixing 20 μl of peptide solutions with 2 μl of 2.2 mM 
tyramine (internal standard for quantitative analysis). Sample 
solutions were injected into the capillary by 20 nbar pressure 
for 10 s. Separations were performed at +25 kV (anode at the 
capillary injection end). The electrode vessels were replenished 
with fresh BGE after each run. All analyses were performed in 
triplelicate. Quantitative analysis was based on the ratio of cor-
corrected (migration time normalized) peak areas of peptides of 
interest and the internal standard (tyramine) (32).

Mass Spectrometry—The analysis of enzymatic cleavage 
products of transmembrane peptides was carried out using 
MALDI-TOF mass spectrometry on an UltraflaXtreme™ MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Ger-
many) with α-cyano-4-hydroxycinnamic acid matrix using a 
thin-layer method (33). For routine quality control during pep-
tide synthesis, mass spectra were acquired on a Waters Micro-
mass ZQ ESCi multidiode ionization mass spectrometer, and 
LTQ Orbitrap XL (Thermo Fisher Scientific) for HR-MS exper-
iments, in both cases using ESI (+) ionization.

Gel-based Assay for Rhomboid Activity—For gel-based assays 
used in Fig. 1, the purified recombinant full-length maltose-
binding protein thioredoxin fusion proteins harboring the 
transmembrane domains of TatA, LacYTM2, Gurken, and 
Spitz (18) were used as substrates. The reaction was carried out in 
50 mM Tris, pH 7.4, 100 mM NaCl, 10% (v/v) glycerol, 0.05% 
(w/v) DDM, and 5 μM substrate. Enzyme concentrations varied 
to ensure adequate conditions for measurement of initial reac-
tion rates for each enzyme-substrate combination. Time 
courses were measured by withdrawing 10-μl aliquots from the 
reaction mixture after 10, 20, 30, 40, 50, 60, and 120 min from 
the start of the reaction, and stopping the reaction by the 
addition of SDS-PAGE sample buffer. The reaction mixtures 
were analyzed by SDS-PAGE, Coomassie staining (Instant-
Blue, Expeodon, UK), and densitometry as described (19), 
and initial reaction rates were converted to molar catalytic 
activities defined as the number of substrate molecules con-
verted by a molecule of the enzyme per unit of time (consis-
tent with the definition by IUPAC (34, 35)). Variations in 
conditions used for measurements in Fig. 6 are denoted in the 
figure legend.

The in vivo assay of rhomboid activity was carried out essen-
tially as described (19). Cleavage products were detected by 
SDS-PAGE and Western blotting using primary anti-penta-His 
mouse monoclonal antibody (Thermo) and IRDye 800CW goat 
anti-mouse fluorescent secondary antibody (LiCor). Densitom-
etry was done in ImageStudio software (LiCor) and substrate 
conversion (α) was calculated from band intensities as 
\[ \alpha = \frac{[P]}{[S] + [P]} \], where [P] and [S] are product and substrate con-
centrations at time t, which are proportional to the fluores-
cence intensity of the product and substrate bands at time t, 
because the monoclonal antibody binds to the substrate or 
product in a constant molar ratio irrespective of their molecular 
weights.

Fluorescence Assay for Rhomboid Activity—The fluorescence 
assay of rhomboid activity was performed at 37 °C in 96-well 
black HTS plates (Greiner Bio-One). The reaction conditions 
were typically as follows: 20 mM HEPES, pH 7.4, 150 mM NaCl, 
0.05% (w/v) DDM, 12% (v/v) DMSO, and 10 μM fluorogenic 
peptide substrate in a final volume of 50 μl, unless noted oth-
erwise. Concentrations of stock solutions of peptide sub-
strates and inhibitors (if applicable) were determined by 
quantitative amino acid analysis. Fluorescence was read con-
tinuously in a plate reader (Tecan Infinite M1000). Excita-
tion and emission wavelengths were 335 and 493 nm, respec-
tively, for the EDANS-DABCYL substrate, and 553 and 583 
nm for the TAMRA-QXL610 substrates. Data were evalu-
ated in i-Control (Tecan), Excel (Microsoft), GraphPad 
Prism 7 (GraphPad Software, Inc.), and GraFit 7 (Erithacus 
Software, Ltd.) software.

Inhibition Assays—The inhibition assay was carried out in 20 
mM HEPES, pH 7.4, 150 mM NaCl, 12% (v/v) DMSO, 0.05% 
(w/v) DDM at 37 °C in 96-well black HTS plates (Greiner Bio-
one). Purified recombinant full-length GlpG (0.4 μM) was pre-

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incubated with each inhibitor at different concentrations for 1 h at 37 °C. The cleavage reaction was started by adding 10 μM Ksp76 and fluorescence was read continuously to measure initial reaction rates as described above.

Reconstitution into Liposomes—E. coli polar lipids (20 mg), with optionally 0.1 mg of Lissamine Rhodamine B-labeled phosphatidylethanolamine (16:0) (Avanti Polar Lipids) added for visibility, were dried in a glass test tube by manual rotation under a nitrogen stream. Residual traces of solvent were removed by overnight incubation in a vacuum chamber (Binder). The resulting lipid film was hydrated in 5 ml of 50 mM acetate, 150 mM NaCl, pH 4.0, by 2 min vortexing followed by a 1-h incubation in a horizontal shaker at 200 rpm and 37 °C, and 3 cycles of freezing in liquid nitrogen and thawing in a 37 °C water bath. The lipid suspension was then extruded through a 200-nm pore membrane by 19 strokes in an Avanti Mini Extruder (Avanti Polar Lipids).

For reconstitution of proteins and peptides into liposomes, these unilamellar LUVs were solubilized in DM to a final ratio 200-nm pore membrane by 19 strokes in an Avanti Mini JEM-1011 device at 80 kV beam acceleration voltage. Detergent was removed by overnight dialysis against 50 mM phosphate buffer at the indicated concentration and lamellarity. These final proteoliposomes were harvested by ultracentrifugation (250,000 g for 1 h at 4 °C), and resuspended in 10 mM HEPES, pH 7.4, 150 mM NaCl to a concentration of about 33 mg/ml of lipids. The morphology and size distribution of proteoliposomes was analyzed by electron microscopy.

Transmission Electron Microscopy—Liposome samples were negatively stained with 2% phosphotungstic acid on carbon-coated electron microscopy grids and analyzed with a JEOL JEM-1011 device at 80 kV beam acceleration voltage.

CD Spectroscopy—Protein and peptide samples were dissolved in 50 mM phosphate buffer at the indicated concentrations and in the presence of detergent as indicated, or reconstituted in LUVs made of E. coli polar lipids and extruded by 100-nm filters to minimize light scattering. Electronic circular dichroism spectra were collected by a Jasco 815 spectrometer (Tokyo, Japan) in the spectral 195–280 nm range using a cylindrical 0.02-cm quartz cell with 0.1-nm step resolution, 5 nm/min scanning speed, 16 s response time, and 1 nm spectral band. After baseline correction, the spectra were expressed as molar ellipticity per residue θ (deg cm² dmol⁻¹). Numerical analysis of the secondary structure and secondary structure assignment were performed using a CDPro software package and CONTIN program (36, 37).

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Author Contributions—K. S. conceived and coordinated the study, designed experiments, and wrote the paper with the input of A. T., M. I., S. S., J. B., J. S., M. R., and V. K. S. S. and P. M. designed and S. S. performed all chemical syntheses. M. R. and V. K. designed and performed all capillary electrophoresis analyses. M. I. analyzed kinetics data, R. H. performed electron microscopy, and L. B. designed and performed all circular dichroism measurements. J. B. performed and evaluated experiments shown in Fig. 6, A and B. J S performed and evaluated experiments shown in Fig. 4, A, D, and E. K. Š designed, performed, and analyzed data shown in Fig. 1A. P. R. and E. P. contributed to experiments shown in Fig. 6, A, B, and D. L. P. established the fluorogenic assay and performed and evaluated experiments shown in Fig. 1D. J. Brezničová contributed to all mass spectrometry experiments, and A. T. designed, performed, and evaluated all other kinetics and inhibition measurements that are the basis of this manuscript.

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Sensitive Versatile Fluorogenic Transmembrane Peptide Substrates for Rhomboid Intramembrane Proteases

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