Both positional and chemical variables control in vitro proteolytic cleavage of a presenilin ortholog

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Running Title: IAP substrate specificity

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ABSTRACT

Mechanistic details of intramembrane aspartyl protease (IAP) chemistry, which is central to many biological and pathogenic processes, remain largely obscure. Here, we investigated the in vitro kinetics of a microbial intramembrane aspartyl protease (mIAP) fortuitously acting on the renin substrate angiotensinogen and the C-terminal transmembrane segment of amyloid precursor protein (C100), which is cleaved by the presenilin subunit of γ-secretase, an Alzheimer disease (AD)-associated IAP. mIAP variants with substitutions in active-site and putative substrate gating residues generally exhibit impaired, but not abolished, activity toward angiotensinogen, and retain the predominant cleavage site (His–Thr). The aromatic ring, but not its hydroxyl substituent, in Tyr of the catalytic Tyr–Asp (YD) motif plays a catalytic role, and the hydrolysis reaction incorporates bulk water as in soluble aspartyl proteases. mIAP hydrolyzes the transmembrane region of C100 at two major presenilin cleavage sites, one corresponding to the AD-associated Aβ42 peptide ( Ala–Thr) and the other the non-pathogenic Aβ48 (Thr–Leu). For the former site, we observed more favorable kinetics in lipid bilayer-mimicking bicelles than in detergent solution, indicating that substrate-lipid and substrate-enzyme interactions both contribute to catalytic rates. High-resolution MS analyses across four substrates support a preference for threonine at the scissile bond. However, results from threonine-scanning mutagenesis of angiotensinogen indicate a competing positional preference for cleavage. Our results indicate that IAP cleavage is controlled by both positional and chemical factors, opening up new avenues for selective IAP inhibition for therapeutic interventions.

INTRODUCTION

Intramembrane proteases (IPs) cleave within a transmembrane (TM) helix of membrane-bound substrates to release cytoplasmic or extracellular proteins/peptides, which in turn translocate to different regions of the cell where they elicit their corresponding biological response related to, for example, cell differentiation, development, and metabolism (1). Despite their broad biomedical reach, basic questions surrounding the structure of the active enzyme, how substrates are presented, and how hydrolysis chemistry occurs in an active site sequestered within the hydrophobic lipid membrane, remain active areas of research.

The least biochemically understood IP is the intramembrane aspartyl protease (IAP) enzyme class, one of just three bona fide IP types that hydrolyze substrates within the hydrophobic lipid environment by using different catalytic nucleophiles (2). IAPs employ membrane-
embedded aspartate residues found in the consensus sequence motif YD...GxGD (where x is any amino acid, typically L or M) to catalyze peptide hydrolysis in the lipid membrane. The eukaryotic IAP signal peptide peptidase (SPP) acts on leader sequences found in nascent polypeptides (3,4). N-terminal signal sequences are first cleaved in the endoplasmic reticulum from a maturely folded protein by the soluble enzyme signal peptidase. The membrane-embedded signal peptide is then further processed by SPP to liberate shorter remnants from the membrane, which become signaling peptides. The known biological substrates of human SPP include the leader peptides of proteins involved in innate immunity (5), inflammatory response (6), and Hepatitis C viral replication (7). SPP also shares key sequence similarity with presenilin, the catalytic component of the γ-secretase complex involved in the production of amyloid-β (Aβ) peptides implicated in Alzheimer disease (8). Presenilin and SPP both contain membrane-embedded active site motifs (Fig. 1A), can cleave the same substrates, and are inhibited by the same active site-directed small molecules (9). These structural and catalytic similarities extend to IAP orthologs across the domains of life (10-12).

Despite intense work in the area, how IPs distinguish substrates from non-substrates and thus avoid deactivating a significant percentage of the proteome is unclear (13). There is no consensus motif across the ~90 different substrates reported for γ-secretase or the ~30 for SPP (14,15). Substrate sequences exhibit high variability in amino acid sequence and length (16,17). Early studies suggested a requirement for helix-breaking residues within the TM substrate at or around the cleavage site. Such residues were proposed to destabilize the TM helix substrate and thus serve as a driving force for exposure of the scissile bond to the protease (18-20). However, canonically helical residues, e.g. Leu, have been reported at the cut site of IAP substrates (10,11). In such cases, access to the scissile bond should be restricted leaving the driving force for hydrolysis ambiguous. Conversely, residues other than aspartate within the IAP enzyme that affect catalysis – kinetics, cleavage specificity, substrate and water entry – are largely obscure. For example, the specific roles of Tyr and Gly within the highly conserved YD...GxGD motif remain to be clarified. Finally, recent structures (21,22) do not reveal the site of substrate or water entry. The TM helices proximal to the proposed C-terminal substrate-gating motif PAL in presenilin (Fig. 1A) (23-25) need to undergo a substantial conformational change to enable entry of an exogenous TM substrate to the active site, a motion that is chemically incompatible with the potential presence of water in the active site.

Here, we compare in vitro kinetic parameters and cleavage sites of a microbial IAP ortholog (mIAP) from Methanoculleus marisnigri toward two substrates, one fortuitous (angiotensinogen cleaved by renin, a soluble aspartyl protease) (26) and one biological (isolated regions of amyloid precursor protein C-terminal 100 residues cleaved by γ-secretase, C100). Our studies reveal a preference for a polar residue, Thr, at the scissile bond across all substrates. Using the angiotensinogen sequence, we examined biochemical properties of YD...GxGD and PAL motif variants. Kinetics are impaired except in the case of Y161F, and the predominant His-Thr cleavage site (26) is retained. mIAP cleaves the C100 sequence at Ala-Thr, the so-called γ-secretase ‘γ-site’ (27) leading to the formation of Alzheimer-associated amyloid-β species, Aβ42 (28). Kinetics are more favorable in bicelles than in detergent solution, and both conditions lead to faster kinetics than the reaction with the angiotensinogen substrate. mIAP also cleaves the so-called γ-secretase ‘ε-site’ corresponding to longer Aβ peptides (27), centered around Thr. However, Thr-scanning mutagenesis within the renin substrate reveals cleavage is preferred at the original cut site, even absent a Thr side chain. Our systematic biochemical study demonstrates an interplay between chemical and positional preferences for cleavage. Such results provide a foundation for elucidating substrate entry and other mechanistic details to better enable the development of selective inhibitors for diseases associated with IAP catalysis, such as Alzheimer and Hepatitis C viral infection, without affecting processing of substrates for other biological processes.

RESULTS
Enzymatic analysis of mIAP mutants.
To assess contributions of particular residues to catalysis (Table 1, Fig. 1A, see below), we
employed a continuous kinetics Förster resonance energy transfer (FRET) peptide assay in combination with a discontinuous gel-based assay, methodology we reported previously (26). Kinetic assays with mIAP variants were conducted using the fortuitous renin FRET peptide (Ren390FRET, HPPFHVLVIHT sequence flanked by Arg-Glu(5-[(2-aminomethyl)amino]naphtalene-1-sulfonate (EDANS fluorophore)- and Lys([4-(4-dimethylamino)phenyl]azo)benzoic acid (DABCYL quencher)-Arg). Due to the incompatibility of the FRET assay components with mass spectrometry analysis, we turned to a gel-based assay that uses a fusion protein (MRS$_{WT}$) in which the renin substrate sequence HPPFHVLVIHT from angiotensinogen is flanked by E. coli maltose binding protein (MBP) and yeast small ubiquitin-like modifier (SUMO). Cleavage profiles from a gel-based assay are analyzed by liquid chromatography tandem mass spectrometry (LC-MS) analysis (26). In aqueous solution, MRS$_{WT}$ adopts an elongated structure as visualized by the small angle X-ray scattering (SAXS) envelope (Fig. 1B, Supporting Information (SI) Fig. S1), rendering the desired 10-residue angiotensinogen sequence (HPPFHVLVIHT$_{10}$) accessible to detergents and mIAP.

mIAP variants (Table 1) Y161A, Y161F, and G219A within the YD$_{162}$...GxGD$_{220}$ motif and Q272A, L275F on the C-terminal helix near the presenilin PAL motif were purified and biophysically characterized as for wild-type (WT). Like WT, all mIAP mutants exhibit α-helical signatures (SI Fig. S2). Although the thermal melts did not yield a sigmoidal curve, both WT and mutants display similar curves, indicating relative structural characteristics are retained upon introducing mutations.

Kinetic and cleavage site profiling reveal that mIAP is largely tolerant to mutation, with primary defects observed in catalytic efficiency and details of processivity. The only variant tested that exhibits Michaelis-Menten kinetic properties indistinguishable from WT is mIAP$_{Y161F}$, representing the Tyr in the Y$_{161}$D catalytic motif (Fig. 1C, Table 1). This result indicates that the phenyl ring, but not the hydroxyl group, is the relevant chemical feature needed for catalysis. This result is surprising given the dearth of hydrogen bond donor/acceotors in the active site that might be capable of activating water or stabilizing an anionic tetrahedral intermediate during hydrolysis. In support for the importance of the phenyl ring, catalysis by mIAP$_{Y161A}$ is impaired, with a reduced Vmax and two-fold lower catalytic efficiency (Fig. 1C, Table 1). The catalytic motif mutant, mIAP$_{D219A}$, adjacent to the aspartate in GxG$_{219}$D, and the site of the familial Alzheimer disease (FAD) mutant G384A in presenilin (29,30), exhibits catalytic rates similar to mIAP$_{Y161A}$ (Table 1). Previous studies of the G384A presenilin variant (31) and corresponding variant in other SPP homologs (11,32) detected reduced end product via immunoblot. Consistent with our measurements (Table 1), one additional study of the G384A presenilin variant reported two-fold reduced Vmax compared to WT enzyme (33). While rigidifying this region with the G219A substitution is detrimental to optimal orientation of Asp$_{220}$ for catalysis, it is noteworthy that the reaction still proceeds.

In terms of how substrate may gain entry into the active site, replacing the mIAP sequence AGL$_{275}$ with PAL$_{435}$ found in presenilin (23-25) reduces catalytic efficiency by ~80% compared to WT, indicating PAL is not a favorable substitute (Fig. 1D, Table 1). For mIAP$_{L275F}$, corresponding to the presenilin L435F FAD mutant (34-36), catalytic efficiency is similar to WT, but this is due to the counteracting effect of a ~60% decrease in Vmax combined with 50% improvement in Km (Fig. 1D, Table 1). More favorable substrate binding to the enzyme when the aromatic Phe is present appears to be unfavorable to some aspect of catalysis (see below). For mIAP$_{Q272A}$, immediately N-terminal to AGL$_{275}$, catalytic efficiency is half of WT due to decreased Vmax. Km remains near that of WT (Fig. 1D, Table 1), so this residue appears not to modulate substrate binding and may instead play a cursory role in positioning other helices for catalysis.

Given the sensitivity of our FRET assay in detecting low levels of cleavage for the aforementioned variants, we tested the hypothesis that polar Asn in place of the negatively charged Asp residues supports catalysis. Each of the catalytic mutants, mIAP$_{D162N}$, mIAP$_{D220N}$, and the double mutant (DM) mIAP$_{D162N/D220N}$ were found to be inactive (Fig. 1E). Thus, the specifically charged state(s) of the aspartic residues are critical for mIAP proteolysis.
Variants of mIAP exhibiting measurable activity in the FRET assay cleave MRS\textsubscript{WT} (Fig. 1F) with profiles that mirror WT mIAP, namely, a predominant cut site at His\textsubscript{9}-Thr\textsubscript{10} (Fig. 1G, H, SI Table S1). However, subtle differences in overall cleavage profiles suggest that members of the YD...GxGD and PAL sequence motifs proximal to the catalytic aspartates (Fig. 1A) influence processivity of IAP cleavage when presented with the renin substrate. For example, for WT-like mIAP\textsubscript{Y161F}, cleavage at Thr\textsubscript{10}-Met\textsubscript{11}, the second-most preferred site for WT and beyond the sequence harbored by Ren390FRET, is substantially diminished (Fig. 1G). In addition, mIAP\textsubscript{L275F} displayed higher cleavage site specificity than WT, with little to no product at positions His\textsubscript{5}-Leu\textsubscript{6} or Ile\textsubscript{8}-His\textsubscript{9}. Thus, tighter substrate binding by mIAP\textsubscript{L275F}, reflected in the lower Km value in the FRET assay, appears to be detrimental to processivity.

**Role of bulk water in IAP catalysis.** In the case of soluble aspartyl proteases, water is activated for nucleophilic attack (37). To test for incorporation of bulk water in IAP catalysis, we carried out the MRS\textsubscript{WT} cleavage reaction in the presence of 75% H\textsubscript{2}\textsuperscript{18}O. LC-MS analysis of the resulting cleavage products reveals robust \textsuperscript{18}O incorporation at the carboxy terminal of the N-terminal cleavage products, exceeding the observed \textsuperscript{16}O incorporation in the expected proportion (Fig. 1I). To our knowledge, this isotope partitioning experiment is the first to provide direct evidence for a role for bulk water in an IAP.

**mIAP cleavage of the presenilin C100 substrate in the ‘γ-site’ region.** Kinetic analysis of mIAP was expanded next to a second substrate, C100FRET, which contains 8 residues surrounding the ‘γ-site’ from the γ-secretase C100 substrate (GGVVIATV flanked by N-methyl anthranilate (Nma) fluorophore and lysine-modified with 2-nitrophenol (DNP) quencher, followed by triple D-Arg), associated with Aβ42 production. With this substrate, the initial velocity is sustained over the first 2 h of the incubation, and arbitrary fluorescence units were converted to product concentration with a calibration curve using free Nma (SI Fig. S3).

Reactions of mIAP and C100FRET were conducted in n-dodecyl-β-D-maltoside (DDM) and bicelles composed of 3-((3-cholamidopropyl)dimethylammonio)-2-hydroxy-1-propanesulfonate (CHAPSO) and 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) (38). mIAP cleaves C100FRET with similar V\textsubscript{max} in detergent and in bicelles, but Km is notably more favorable in bicelles (Fig. 2A, Table 2). As expected, catalytic mutants mIAP\textsubscript{D162A}, mIAP\textsubscript{D220A}, and DM mIAP\textsubscript{D162A/D220A} failed to cleave C100FRET (Fig. 2A) and 1,3-di-(N-carboxybenzoyl-L-leucyl-L-leucyl)amino acetone ((ZLL)\textsubscript{2}ketone) is a competitive inhibitor of WT mIAP, with the Ki value we reported before (26) (Fig. 2B). For reactions in DDM, V\textsubscript{max} is nearly four-fold faster and Km is 20% lower for C100FRET compared to Renin390FRET in either DDM or bicelles, resulting in ~5-15-fold higher catalytic efficiency (Tables 1, 2). The dramatic kinetic difference is consistent with the fact that C100FRET derives from a biological substrate whereas Ren390FRET, while competent for catalysis, is not a biological IAP substrate. Overall, our Km is in the typical low micromolar range, similar to the soluble aspartyl protease renin (39), but mIAP is less efficient than renin in hydrolyzing substrates. Our data are consistent with other reports that IAP cleavage is sluggish, but it is challenging to further compare our study to analogous ones of γ-secretase. For example, experiments using C100FRET to study γ-secretase kinetics did not convert to an absolute scale (40,41). In other studies, where initial rates were evaluated via immunoblot quantitation after a set incubation time, our Km is either 10-fold weaker (33) or 30-fold tighter than that of γ-secretase (42).

To identify the cleavage site within the C100FRET sequence, we adapted our gel assay by generating MCS10, an analogous 10-mer substrate in which the intervening sequence between MBP and SUMO in MRS\textsubscript{WT} was converted to G\textsubscript{17}GVVIATV\textsubscript{146}. MCS10 contains the octapeptide sequence of C100FRET plus IV\textsubscript{46} from C100. An increase in the N-terminal product was observed over the course of a 2-24 h reaction period in DDM (Fig. 2C, SI Table S2). In parallel with results from C100FRET, the reaction between mIAP and MCS10 in bicelles was faster than in DDM, as product was readily visible within 30 minutes followed by a continued increase over the duration of the experiment (Fig. 2D). As expected, the reaction is dependent on IAP concentration, is inhibited by (ZLL)\textsubscript{2}ketone,
and catalytic mutants mIAP\textsubscript{D162A}, mIAP\textsubscript{D220A} and DM mIAP\textsubscript{D162A/D220A} are unable to cleave MCS10 (Fig. 2E-G). LC-MS cleavage profiles for MCS10 reveal highly specific cut sites – Ala\textsubscript{42}-Thr\textsubscript{43} and Thr\textsubscript{43}-Val\textsubscript{44} – both in DDM and bicelles (Fig. 2H). Isotope partitioning again demonstrates the use of bulk water in the mIAP reaction (SI Fig. S4). In sum, like results for mIAP cleavage of the MRS\textsubscript{WT} substrate, these data indicate the involvement of a site-adjacent Thr residue using a typical protease reaction mechanism.

The identified cleavage positions for mIAP reacted with MCS10 recapitulates the \(\gamma\)-secretase \(\gamma\)-site cut site leading to Alzheimer-associated A\textbeta{42} and A\textbeta{43} production, but we did not observe even low-level background cleavage at Val-Ile corresponding to the \(\gamma\)-secretase cleavage product A\textbeta{40} (27). On the basis of the MRS substrate (above and see below), the MBP fusion protein should not be restricting access to the A\textbeta{40} cut site. Our results differ from a previous study indicating that the same mIAP ortholog cleaves an alternately epitope-tagged C100 to release predominantly A\textbeta{38} and A\textbeta{40}, with only a minor product of A\textbeta{42} (12). In that study, kinetics were not investigated, and detailed biochemical characterization of the substrate was not provided. Further work will be required to better assess why cleavage at sites generating A\textbeta{38} and A\textbeta{40} were not observed in our system.

**mIAP cleavage of the presenilin C100 substrate in the ‘\(\epsilon\)-site’ region.** Prior to cleavage at the \(\gamma\)-site, presenilin has been shown to cleave at the C100 ‘\(\epsilon\)-site’, which generates A\textbeta{48}-49 (27). After unsuccessful attempts to generate a fusion substrate containing both sites (MBP-G\textsubscript{37}GVVIATVIVITLVM-SUMO, MRS15) due to substrate aggregation and truncation issues (SI Fig. S5), we studied mIAP cleavage of the isolated \(\epsilon\)-site by two fusion substrates in shifted register, MCSTV (MBP-T\textsubscript{45}VIVITLVM-SUMO) and MCSGG (MBP-GG-V\textsubscript{44}IVITLVM-SUMO) (Fig. 3A). MCSGG includes the flexible Gly-Gly hinge thought to help position the \(\epsilon\)-site in the active site of \(\gamma\)-secretase (43). mIAP cleaves both substrates (Fig. 3B, SI Table S2) and LC-MS analyses converge on cut sites at three consecutive peptide bonds. The predominant cut site is Thr\textsubscript{48}-Leu\textsubscript{49} (Fig. 3C, D), which corresponds to the presenilin-associated product A\textbeta{48}. Whereas the Thr residue of MCS10 \(\gamma\)-site and MCSGG \(\epsilon\)-site are in register (Fig. 3C), MCSTV and MCSGG are offset (Fig. 3D). This result suggests that sequence preference for Thr may preside over positional preference relative to the membrane.

**Probing substrate specificity by Thr-scanining within the MRS substrate.** To further address the interplay between chemical and positional preferences for mIAP cleavage, we introduced Thr substitutions adjacent to His residues in MRS\textsubscript{WT} (IHP,FHL\_6VIHT\_10), namely, replacing P\textsubscript{3} or L\textsubscript{4} with Thr, and either retaining or removing the original His\textsubscript{\(\gamma\)}-Thr\textsubscript{10} cut site (MRS\textsubscript{PTT}, MRS\textsubscript{PTT/T10L}, MRS\textsubscript{L6T}, MRS\textsubscript{L6T/T10L}, Fig. 4A and (26)). Since cleavage at His\textsubscript{\(\gamma\)}-Leu\textsubscript{6} was of low abundance in MRS\textsubscript{WT}, we predicted that replacing Thr\textsubscript{10} in MRS\textsubscript{WT} with Leu (MRS\textsubscript{T10L}) would disfavor this position as a cleavage site, and serve as a control for positional preference (Fig. 4A). mIAP cleaves all five MRS Thr-scanining mutants, including MRS\textsubscript{T10L} where no H-T is present (Fig. 4B-E, SI Table S2). The major cleavage position remains the same as for MRS\textsubscript{WT}, and does not preferentially shift internally for MRS\textsubscript{LT6}, MRS\textsubscript{L6T/T10L} (Fig. 4D), MRS\textsubscript{PTT} or MRS\textsubscript{PTT/T10L} (Fig. 4E). Thus, in contrast with C100 \(\gamma\)- and \(\epsilon\)-site cleavages, positional preference presides over chemical preferences in this sequence.

**DISCUSSION**

Intramembrane proteolysis must be highly regulated in the cell (13), yet contrary to intuition and knowledge of soluble proteases (44,45), data to date do not converge on recognition motifs for IPs within their TM substrate(s). Cleavage of non-physiological substrates has been demonstrated for IAPs (10-12,46). While helix-breaking residues within the TM segment were once thought to be important for cleavage (18,20,47), examples to the contrary appear in the literature (10,11). Confounding the puzzle for IAPs is that IAPs appear to cleave at multiple cut sites, and/or trim TM helices to smaller segments that can eventually be released from the membrane (27,48-50). Systematic in vitro studies using purified model enzymes should help clarify the physicochemical preferences of IAPs toward their substrates.

Overall, our results point to faster kinetics and a higher degree of specificity for IAP cleavage than previous studies have reported using other
IAP substrate specificity

methods. Across the substrates examined, a chemical preference for Thr at the scissile bond emerged, regardless of enzyme or substrate variant used. None of the residues targeted for mutagenesis within mIAP substantially altered cleavage profiles, even where kinetic parameters that might be correlated with specificity, such as Km, were altered. Processivity, largely confined to residues adjacent to Thr on the substrate, were altered in some of the mIAP variants. In contrast to the fortuitous cleavage of the renin substrate, mIAP cleaved regions of the presenilin C100 substrate with a high degree of specificity around Thr. Finally, bulk water is incorporated into the product of the hydrolysis reaction, confirming that the chemical reaction is similar to that of soluble aspartyl proteases (37), but how water enters the active site, and the extent to which each Asp functions in a manner similar to those in soluble aspartyl proteases, remain open questions for future work.

Activity of mIAP toward C100FRET is five-fold more efficient than the fortuitous Ren390FRET, reflected in faster Vmax and lower Km. There is no difference in cleavage profiles of MCS10 in bicelles or DDM indicating that the presentation of MCS10 to mIAP within DDM micelles and bicelles, and subsequent cleavage processes, are similar. Yet, the Km is significantly more favorable for the reaction in bicelles, which contains CHAPSO, a cholesterol mimic. Cholesterol is a modulator of both membranes (51,52) and C100 catalysis (53-57). Indeed, relevant regions of C100 are partitioned well inside the lipid bilayer and specifically bind cholesterol (53).

Our study delineates properties and/or roles for specific residues on the enzyme beyond the two catalytic Asps. The bulky phenyl ring, but not the hydroxyl group, in the YD162 motif on TM helix 6 is the pertinent chemical property, perhaps to orient the substrate in the active site, to stabilize residues from another TM helix in the conformation that supports bound substrate. Reinforcing the need for an aromatic residue, but not a proton donor/acceptor at this position, inspection of hundreds of presenilin-like protein sequences (PFAM PF01080 (58)) reveals WD is a prevalent alternative to YD. Likewise, in the C-terminal TM helix 9, both mIAPA273F/G274A and mIAPL275F within the PAL motif exhibit slowed kinetics, but Km values are as favorable, or moreso, than for WT. In our pseudo first order reaction setup, the Km reflects all of the binding events prior to catalysis, which is the slow step. Thus, since the substrate has higher affinity for the mutant enzyme than WT mIAP, impaired hydrolysis rates reflect a problem later in the process, for example, enabling a conformational change or correctly orienting the substrate for optimal cleavage(s).

The finding that mIAP generally exhibits a sequence preference for Thr at the scissile bond is consistent with numerous studies of γ-secretase and C100 describing γ- and ε-site cleavages, but the specificity with which mIAP cleaved at these sites containing a Thr across several substrates was unexpected with respect to the general tendency IAPs exhibit toward processivity. Thr exhibits average helical propensity (59-61), but intrahelical hydrogen bonds from the Thr side chain induces a more significant bend (62) and thus can displace remaining residues traversing the lipid membrane. Other residues capable of forming intrahelical hydrogen bonds include Ser and Cys (62). TM segments of SPP substrates (preprolactin, HLA-A*0301, and Hepatitis C virus), and γ-secretase substrates (Erbb4, Notch-1, p75, Delta, Jagged, and STIM1 (5,7,63-68)), harbor Thr, Ser, and Cys residues, but most are not obviously in register with Thr residues in C100. As demonstrated by the Thr-scanning experiment, which failed to significantly shift to internal H-T sites, positional preferences relative to the membrane are also relevant, perhaps due to substrate interactions with the membrane. Notably, in the case of Notch-1, the relative position of presenilin cleavage, V-L (69), is similar to that of T49-L from C100 (53) with respect to the membrane bilayer. Our data point to a likelihood that different IAP substrates are governed by disparate physicochemical properties. Broadening such studies to other substrates and IAP family members should further clarify substrate preferences, ultimately leading to the ability to better target substrates selectively for therapeutic applications.

EXPERIMENTAL PROCEDURES

Molecular Biology. The gene sequence for mIAP was cloned into the pet 22b(+) (Novagen) vector with C-terminal hexa-histidine tag as...
described previously (26). MRSWT was cloned in pEX-K vector by MWG Operon. Substrates MCSTV (MBP-TVIVITLVML-SUMO) and MCSGG (MBP-GGIVITLVML-SUMO) were cloned into pMAL-c4x and purchased from GenScript. Mutations to mIAP, MRS substrates (to MCS10 and Thr-scanning variants), and MCS (e.g. to generate MCS15) were carried out via site-directed mutagenesis (Agilent QuickChange Lightning kit, primer sequences in SI Table S3) and verified by DNA sequencing (MWG Operon).

Expression and purification of enzyme variants and fusion substrates. Plasmids containing mIAP (WT and variants) were transformed into E. coli Rosetta 2 cells (Novagen). Large scale growth of bacteria, membrane isolation and protein purification steps were carried out as described previously (26), with the following modifications found to improve protein yield: cells were induced with 0.2 mM isopropyl-\(\beta\)-D-1-thiogalactopyranoside (IPTG) at OD\(_{600\text{nm}}\) = 1.5, followed by growth overnight at 22°C. During nickel affinity purification, weakly bound impurities were first removed with 5% Buffer B (50 mM Hepes (pH 7.5), 500 mM NaCl, 500 mM imidazole, 0.1% DDM) before the full gradient was applied. Protein purity after size exclusion chromatography (SEC) on Sephacryl S-300 (GE Healthcare) was assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% polyacrylamide) stained with Coomassie. Pure protein was concentrated and exchanged into PBS, 0.05% DDM using a 15 mL Amicon Ultra MWCO 30K concentrator (Millipore). Protein was measured on a ThermoScientific NanoDrop spectrophotometer using a calculated extinction coefficient, \(\varepsilon = 69,330 \text{ M}^{-1} \text{ cm}^{-1}\) (70).

**FRET assay and inhibitor studies.** Assays with Ren390FRET (Anaspec) were conducted as described previously (26). For C100FRET (Millipore), lyophilized peptide was dissolved in DMSO to generate a stock solution at 1.25 mM. A working stock solution (500 \text{ \mu M}) was prepared by diluting the original stock with 10 mM sodium phosphate, pH 7.2, 150 mM NaCl (PBS), 0.05% (w/v) DDM. The substrate was not reconstituted into bicelles due to phase incompatibility between liquid phases of bicelles and DMSO. The 500 \text{ \mu M} C100FRET working stock was sonicated (Branson ultrasonics corporation) at 50-60 Hz, 117 V, 0.7 Amp for 30 min prior to being diluted to 3-40 \text{ \mu M} solutions with PBS with 0.05% DDM and dispensed into black-bottomed, non-binding 96-well plates (Corning). Plates were covered with optical adhesive film (Micro-Amp) and incubated at 37 °C for 30 min, a process that resolved the initial high background from the C100FRET substrate. Freshly purified mIAP (0.5 \text{ \mu M}, in PBS 0.05% DDM and reconstituted inside 5% (v/v) bicelles (26)) was then added to the substrate solution in the 96-well plate. The fluorescence readings were acquired every 2 min for 2.5 h at 37°C in a Synergy H4 plate reader (BioTek, \(\lambda_{\text{ex}} = 350 \pm 9 \text{ nm}, \lambda_{\text{em}} = 440 \pm 9 \text{ nm}\)). The initial velocity was determined from the fluorescence readings over the first 2 h. The background reading at each
substrate concentration was subtracted and the fluorescence reading was converted from arbitrary fluorescence unit (Afu) to concentration of product (nM) by using the standard calibration curve of free Nma (SI Fig. S3). The inner filter effect was not observed for the Nma-Dnp donor-acceptor pair. GraphPad Prism 5 software was used for Michaelis-Menten kinetic analysis as described previously (26). Twelve replicates from independent batches of cell paste and enzyme purification were used in data analysis.

For inhibitor studies, (ZLL)ketone (Calbiochem) was dissolved in DMSO to prepare 1 mM stock. (ZLL)ketone was diluted to a final 2- to 10-fold molar excess of the enzyme in the activity assay and preincubated with purified enzyme for an hour at 37°C. C100FRET was then added and the assay conducted as described above. Kinetics data of nine independent replicates were analyzed as described above.

_Gel-based assay._ Varying concentrations of mLAP (1-8 µM), prepared in PBS with 0.05% DDM or 5% (v/v) bicelle, were incubated with MCS10 (5 µM) at 37°C for 24 h. For time course experiments, 16 µM of mLAP was incubated with MCS10 (5 µM) and the reaction was quenched at different time points by adding equal volume of Laemmli SDS-PAGE sample loading dye containing β-mercaptoethanol, followed by storage at -20 °C. For experiments with (ZLL)ketone, a range of inhibitor concentration (8-32 µM) was pre-incubated with 16 µM of IAP at 37°C before adding MCS10 (5 µM). The cleavage reaction samples were separated by SDS-PAGE (12% (w/v) polyacrylamide) followed by transfer onto polyvinyl difluoride (PVDF) membrane (MilliPore). Standard Western blot procedures were performed using MBP-probe mouse monoclonal IgG primary antibody (Santa Cruz, 1:1000) and horseradish peroxidase (HRP)-conjugated goat anti-mouse monoclonal IgG secondary antibody (KPL, 1:5000). The PVDF membrane was sprayed with HyGlo Quick Spray (Denville) and visualized on an Amersham Imager 600 (GE Healthcare) except for the blot in Fig. 2C, which was exposed using autoradiography film and visualized on Konica SRX 101A film processor. The molecular mass ruler was PageRuler Plus prestained protein ladder (Thermo Scientific).

For LC-MS analysis, the gel assay was set up as described above except that ice-cold acetone (6.5 x sample volume) was added to quench reaction at 24 h for samples containing MCS10 substrate and at 24 h and 48 h for samples containing MRS substrate, followed by storage at -20 °C. After decanting the acetone, samples were analyzed by SDS-PAGE. LC-MS analysis of samples was carried out as described previously (26) with the following modifications. An UltiMate™ 3000 RSLCnano System UPLC system (Dionex) was coupled to a Q Exactive Plus Mass Spectrometer (Thermo Scientific). The Mascot Search engine (Version 2.6.0) was used with Proteome Discoverer 2.1 (Thermo Scientific). Only peptide spectral matches with an expectation value of less than 0.01 (“High Confidence”) were used.

For isotopic 18O labeling, PBS with 0.05% DDM (75 µL) was first lyophilized in a Speed Vac (Savant) for 30 min, after which 75 µL of heavy water H218O (Cambridge Isotopes) was added. Purified mLAP (16 µM) and either MRSwt or MCS10 (5 µM) was then added to run gel-based assay at 37°C for 24 h. LC-MS analysis of samples was carried out as described above.

_Circular dichroism (CD)._ CD spectra and thermal melts were acquired on a Jasco J-810 spectropolarimeter equipped with Neslab RTE 111 circulating water bath and a Jasco PTC-4245/15 temperature control system using a 0.1-cm cuvette. CD spectra of IAP variants (5 µM in 20 mM HEPES (pH 7.5), 250 mM NaCl, 0.05% DDM) were acquired from 300-200 nm at room temperature. Data were blank-subtracted and converted to molar ellipticity \( \Theta = (\Theta_{\text{obs}} \times 10^6)/(\text{pathlength (mm)} \times \text{c} \times \text{n}) \), where \( \Theta_{\text{obs}} \) is the observed ellipticity (mdeg) at wavelength \( \lambda \); c is the protein or peptide concentration (µM); and n is the number of residues. CD thermal melts were performed with a 1 °C min⁻¹ increase in temperature from 5 to 90°C. Both CD spectra and melts were acquired with 15 averaged scans from 300-200 nm at a 200 nm min⁻¹ scan rate. Data at 222 nm were blank-subtracted, converted to molar ellipticity and plotted against temperature in GraphPad Prism 5.

_SAXS and ab initio modeling._ SAXS data for substrate solutions containing ~1 mg/mL of substrate in 20 mM Hepes (pH 7.5), 250 mM NaCl were collected at 12°C using a Rigaku BioSAXS-
2000 instrument with 2D Kratky collimation and a rotating Cu anode X-ray source (\(\lambda = 1.54187 \, \text{Å}\)). Each sample was exposed for a total of 3 hours using multiple scans to confirm consistency between measurements and the absence of radiation damage. Matched buffers measured at an identical instrument configuration were used for background subtraction. Rigaku SAXSLab software was used for data reduction, and yielded a 1D plot of the scattered intensities over the full range of momentum transfer \(0.0104 < q < 0.6782 \, \text{Å}^{-1}\) [\(q = 4\pi \sin(\theta)/\lambda\), \(\theta\) is the scattering angle and \(\lambda\) is the X-ray wavelength].

Three-dimensional reconstruction of the substrate structure from the SAXS data was performed using the ATSAS software suite (71). Briefly, the pair-distribution function \(P(r)\) (using \(0.0104 < q < 0.3464 \, \text{Å}^{-1}\)) was determined from an indirect Fourier transform of the scattering data for each substrate via GNOM. The DAMMIF tool was then used to rapidly generate 17 compact bead representations of the scattering particle. This initial collection of models was clustered using DAMCUST, and the averaged model from the most populated cluster was used with DAMSTART to generate a fixed core (using a cutoff volume of one half the excluded volume of the particle) for further refinement in DAMMIN. Ten refined models from DAMMIN (slow-mode) were similarly clustered, and a representative model of the most populated cluster was compared to results from a rigid-body model reconstruction. CORAL was employed for rigid-body modeling, performing translation and rotation of high-resolution PDB structure fragments, as well as constructing dummy atom segments of defined lengths linking between domains and at the C-terminus. MBP used for rigid-body modelling was excised from PDB 5CL1, chain A: residues 1-368. The structure for the SUMO domain was taken from the first of 20 conformers in the collection of NMR structures deposited under PDB 1L2N; the first 20 and C-terminal 5 amino acids are absent in the NMR structure. Dummy atom representations of the linker region containing the substrate target sequence, omitted residues of the SUMO domain, and C-terminal hexahistidine-tag were all constructed using CORAL.

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Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contribution

Conceptualization: SHN, SK, MPT, RLL; Data curation: SHN, SK, DMS, HK, RCO, MPT; Formal analysis: SHN, SK, DMS, HK, RCO, VSU, MPT, RLL; Funding acquisition: MPT, RLL; Investigation: SHN, SK, DMS, XT, JBG, APJ, RCO, VSU; Methodology: RCO, VSU, MPT, RLL; Project administration: VSU, MPT, RLL; Supervision: DMS, VSU, MPT, RLL; Validation: SHN, DMS, HK, MPT, RLL; Visualization: MPT, RLL, DMS; Writing-original draft: SHN, MPT, RLL; Writing-review and editing: SHN, SK, MPT, DMS, RLL.
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Table 1. Rationale for mIAP mutation, and associated kinetic parameters for cleavage of Ren390FRET.

| IAP variants | Rationale                                      | Km (µM) | Vmax (nM min⁻¹) | kcat x10⁻³ (min⁻¹) | (kcat/Km) x 10⁻³ (µM⁻¹ min⁻¹) |
|--------------|------------------------------------------------|---------|-----------------|-------------------|-------------------------------|
| WT           |                                                | 7.8 ± 0.7 | 4.1 ± 0.1      | 8.1 ± 0.3         | 1.0 ± 0.1                     |
| Y161A        | YD₁₆₂ motif (active site)                      | 8.9 ± 0.8 | 1.8 ± 0.1      | 3.6 ± 0.2         | 0.40 ± 0.04                   |
| Y161F        | YD₁₆₂ motif (active site)                      | 5.8 ± 0.5 | 3.6 ± 0.1      | 7.2 ± 0.6         | 1.2 ± 0.5                     |
| G219A        | GxGD₂₂₀ (active site)                          | 9.4 ± 0.6 | 2.4 ± 0.2      | 4.8 ± 0.2         | 0.51 ± 0.04                   |
| Q272A        | QAGL₂₇₅ motif (substrate gating)               | 6.2 ± 0.6 | 1.6 ± 0.1      | 3.2 ± 0.1         | 0.52 ± 0.05                   |
| A273P/G274A  | AGL to PAL motif (presenilin substrate gating) | 4.9 ± 0.7 | 0.51 ± 0.04    | 1.0 ± 0.4         | 0.21 ± 0.05                   |
| L275F        | FAD mutation in presenilin; in crystal structure, located between two catalytic aspartates | 3.9 ± 0.5 | 1.5 ± 0.1      | 3.1 ± 0.1         | 0.8 ± 0.1                     |
Table 2. Kinetic parameters for mIAP cleavage of C100FRET in DDM and bicelles, and inhibition by (ZLL)$_2$ ketone.

| Enzyme          | Inhibitor     | Km (µM)       | $V_{max}$ (nM min$^{-1}$) | kcat $\times 10^3$ (min$^{-1}$) | (kcat/Km) $\times 10^3$ (µM$^{-1}$ min$^{-1}$) |
|-----------------|---------------|---------------|---------------------------|----------------------------------|-----------------------------------------------|
| mIAP (DDM)      | -             | 6.0 ± 0.7     | 15.9 ± 0.6                | 32 ± 1                           | 5.3 ± 0.9                                     |
| mIAP (bicelles) | -             | 1.7 ± 0.1     | 13.2 ± 0.2                | 26 ± 1                           | 16 ± 1                                        |
| mIAP            | (ZLL)$_2$ketone|              |                           |                                  |                                               |
| 2x              | 10 ± 1        | 15.8 ± 0.9    | 32 ± 2                    | 3.0 ± 0.7                         |
| 5x              | 13 ± 2        | 15.9 ± 0.9    | 32 ± 2                    | 2.5 ± 0.3                         |
| 10x             | 17 ± 2        | 15.5 ± 0.8    | 31 ± 2                    | 1.8 ± 0.3                         |
Figure 1. Characterization of mIAP mutants. (A) Superposition of mIAP (PDB ID 4HYC, cyan) and presenilin (PDB 5A63 chain B, magenta) structures using secondary structure matching (72). TM helices are numbered from N- to C-terminus. Key sequence motifs highlighted. AS= active site. (B) Ab initio model of MRS\textsubscript{WT} obtained by SAXS. Additional analysis presented in SI Fig. S1. (C, D, E) Michaelis-Menten kinetic analysis of mIAP variant cleavage of Ren390FRET. See also Table 1. (F) Gel assay of mIAP mutants using MRS\textsubscript{WT} substrate. Negative control without enzyme is indicated by (-). The uncut substrate and cleavage products (indicated by arrow) are detected via anti-MBP Western blot. Molecular mass indicated in kDa. (G, H) Cleavage profiles of MRS substrate were generated by Glu-C digestion of the N-terminal product, facilitated by a C-terminal Glu within MBP (black triangle). Major cleavage site is His\textsubscript{9}-Thr\textsubscript{10} (red triangle). Representative LC-MS analysis of MRS\textsubscript{WT} cleavage sites across mutants tested. The relative abundance of each reporter peptide, compared to total peptide spectral matches (PSMs) of seven independent peptide products formed by proteolytic cleavage, is shown. Error bars represent the standard deviation from LC-MS analytical replicates. All LC-MS data for mIAP variants are presented in SI Fig. S6 and Table S1. (I) LC-MS spectrum of reporter peptide (z=3) displaying 350\% more $^{18}$O incorporation than $^{16}$O. (Left) The extracted ion current (XIC) for relative abundance of peptide (ALKDAQTNSIHPFHLVIH) with $^{16}$O incorporation (middle) versus $^{18}$O incorporation at the C-terminal of N-terminal product (right) from enzymatic cleavage. Approximately 11.8\% of peak at 681.7058 is due to two-$^{13}$C isotope of the $^{18}$O-containing peptide.
Figure 2. mIAP cleavage of C100 γ-site. (A) Michaelis-Menten kinetic analysis of C100FRET cleavage by WT mIAP in DDM and bicelles, and by catalytic mutants (D162A, D220A, and DM) in DDM. (B) Kinetic data for mIAP treated with increasing (ZLL)₂ketone. (C-G) Gel assay using MCS10 and mIAP, visualized by anti-MBP immunoblot. (C) Time course in DDM. (D) Time course in bicelles. (E) Product formation as a function of mIAP concentration. (F) Inhibition by (ZLL)₂ketone. (G) mIAP variants D162A, D220A, and DM are not active. For panels C-G, molecular mass is indicated in kDa and negative control without enzyme is indicated by (-). The uncut substrate and cleavage product (indicated by arrow) are detected as in Fig. 1F. (H) LC-MS analysis of MCS10 cleavage sites by mIAP in DDM and bicelles, compared to MRSWT in DDM. Quantification and presentation as in Fig. 1G. All LC-MS data are presented in SI Fig. S7 and Table S2.
Figure 3. mIAP cleavage of C100 ε-site. (A) Overlay of substrates used in this study and relationship to C100 γ- and ε- cleavage sites of γ-secretase. (B) mIAP gel assay using MCSTV and MCSGG substrates, visualized by anti-MBP Western blot. Molecular mass indicated in kDa. (C, D) LC-MS analysis of MCS10, MCSGG and MCSTV cleavage sites by mIAP in DDM. Quantification and presentation as in Fig. 1G. All LC-MS data are presented in SI Fig. S7 and Table S2.
Figure 4. Probing substrate specificity of IAP using MRS Thr-scanning mutants. (A) Sequences generated for this study. (B) Gel cleavage assay using mIAP and MRS substrate variants, visualized by anti-MBP immunoblot. Molecular mass indicated in kDa. (C, D, E) Corresponding LC-MS analysis of reactions from (B) with quantification and presentation as in Fig. 1G. All LC-MS data for MRS substrate variants are presented in SI Fig. S8 and Table S2.
Both positional and chemical variables control in vitro proteolytic cleavage of a presenilin ortholog
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