A common genetic system for functional studies of pitrilysin and related M16A proteases

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Pitrilysin is a bacterial protease that is similar to the mammalian insulin-degrading enzyme, which is hypothesized to protect against the onset of Alzheimer's disease, and the yeast enzymes Axl1p and Ste23p, which are responsible for production of the **a**-factor mating pheromone in *Saccharomyces cerevisiae*. The lack of a phenotype associated with pitrilysin deficiency has hindered studies of this enzyme. Herein, we report that pitrilysin can be heterologously expressed in yeast such that it functionally substitutes for the shared roles of Axl1p and Ste23p in pheromone production, resulting in a readily observable phenotype. We have exploited this phenotype to conduct structure–function analyses of pitrilysin and report that residues within four sequence motifs that are highly conserved among M16A enzymes are essential for its activity. These motifs include the extended metalloprotease motif, a second motif that has been hypothesized to be important for the function of M16A enzymes, and two others not previously recognized as being important for pitrilysin function. We have also established that the two self-folding domains of pitrilysin are both required for its proteolytic activity. However, pitrilysin does not possess all the enzymatic properties of the yeast enzymes since it cannot substitute for the role of Axl1p in the repression of haploid invasive growth. These observations further support the utility of the yeast system for structure–function and comparative studies of M16A enzymes.

Key words: inverzincin, M16A subfamily, pheromone, pitrilysin, yeast mating, zinc-dependent metalloprotease.

INTRODUCTION

The M16A subfamily comprises a group of zinc-dependent metalloproteases whose members are expressed throughout both the prokaryotic and eukaryotic kingdoms [1,2]. M16A proteases have several distinguishing characteristics. They are typically large proteins ($\geqslant$ 100 kDa) that are related by primary sequence. They also contain an inverted zinc-metalloprotease core motif (HXXEH) that is typically located within 200 residues of the N-terminal, as well as a pair of glutamate residues that are located 70 and 77 amino acids distal to the core motif. The conserved residues of this extended metalloprotease motif ($HXXEHX_{69}EX_6E$) are either catalytic, or coordinate the enzyme's catalytic zinc ion and, not surprisingly, these residues are essential for the activity of several M16A subfamily enzymes [3]. However, the biological functions of many M16A enzymes remain largely uncharacterized. While several M16A proteases have been demonstrated to possess *in vitro* proteolytic activity, few native substrates have been reported for members of this subfamily.

The most notable members of the M16A subfamily are the IDE (insulin-degrading enzyme; EC 3.4.24.56), Ptr (pitrilysin; EC 3.4.24.55) and the yeast enzymes Axl1p and Ste23p. These enzymes are highly related in primary sequence. Using human IDE as a reference Ptr, Axl1p and Ste23p have approx. 30, 20 and 40 % identity respectively. This similarity generally extends throughout the entire length of these proteins, but is somewhat higher in the N-terminal half. In addition to the extended metalloprotease motif, four highly conserved motifs can be identified that have a minimum of 50 % identity over a stretch of at least 10 amino acids [4]. One of these four motifs has been proposed (but not yet validated) to be required for the function of M16A enzymes [5]. This study aims to resolve the functional importance of these motifs.

IDE is a mammalian enzyme that is of biomedical interest due to its ability to cleave insulin, the neurotoxic Aβ peptide and other small molecules *in vitro* [6–10]. In animal models, increased expression of IDE is correlated with decreased circulating levels of insulin and Aβ peptide, while IDE deficiency is correlated with increased risks of Alzheimer's disease and Type 2 diabetes mellitus [11–13]. Together, these observations suggest an active role for IDE in the clearance of small peptides. In addition, we have recently demonstrated that IDE can substitute for the activities of the yeast M16A enzymes Axl1p and Ste23p in production of the yeast **a**-factor mating pheromone [4].

Ptr is a secreted protease that is localized to the periplasmic space of *Escherichia coli* [10,14]. It is the founding member of the M16 family, and it has been named previously protease III and protease Pi among others. *In vitro* studies have demonstrated that this non-essential enzyme can cleave insulin, glucagon, Aβ peptide, β-galactosidase fragments and certain short synthetic peptides [10,15]. However, these substrates have been used as surrogate substrates for Ptr, since its physiological targets are unknown.

Ptr is the only M16A protease whose structure has been determined (PDB ID 1Q2L), albeit only recently [16]. This revealed that the enzyme has two nearly identically sized concave domains that are for the most part structurally similar and are tethered by a linker region of 20–30 residues, giving the enzyme the overall appearance of a clamshell. The MPP (mitochondrial processing peptidase) and other enzymes of the distantly related M16B subfamily have a similar appearance, except they consist of two separately encoded domains that are typically referred to as

Abbreviations used: Axl1p–HA, haemagglutinin-epitope tagged Axl1p; IDE, insulin-degrading enzyme; MALDI–TOF-MS, matrix-assisted laser-desorption ionization–time-of-flight MS; MPP, mitochondrial processing peptidase; MSM, multiple-site mutant; Ptr, pitrilysin; Ptr–HA, HA epitope-tagged Ptr; SC, synthetic complete; SD, synthetic dextrose; SSM, single-site mutant; Ste23p–HA, HA-epitope tagged Ste23p; YEPD, yeast extract peptone dextrose.

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Table 1 Yeast strains used in this study

Strain	Genotype*	Reference
IH1783	*MAT***a** *trp1 leu2 ura3 his4 can1*	[20]
IH1793	*MAT*α *lys1*	[20]
RC757	*MAT*α *sst2-1 his6 met1 can1 cyh2*	[48]
Sy3687	*MAT*α *ura3 his3::ura3*	[30]
Sy3721	*MAT*α *ura3 his3::ura3 axl1::HIS3*	[30]
Y272	*MAT***a** *trp1 leu2 ura3 his4 can1 axl1::LEU2 ste23::LEU2*	[17]

*IH1783, IH1793 and Y272 are isogenic, as are Sy3687 and Sy3721.

Table 2 Plasmids used in this study

The list shows the genetic marker used to select the respective plasmids, the promoter used to drive expression of the transgene and the wild-type or mutated protein encoded by the transgene. P_{PGK}, phosphoglycerate kinase promoter.

Plasmid	Description	Reference
pCDK35	Ptr	[22]
pRS316	*CEN URA3*	[23]
pSM463	2μ *TRP1 MFA1*	[18]
pSM703	2μ *URA3* P_{PGK}	[49]
pWS371	*CEN URA3* Axl1p–2HA	[17]
pWS482	*CEN URA3* Ste23–2HA	[17]
pWS603	2μ *URA3* P_{PGK}-Ptr	This study
pWS604	2μ *URA3* P_{PGK}-Ptr–HA	This study
pWS617	*CEN URA3* Ste23–HA	This study
pWS618	*CEN URA3* Axl1p–HA	This study
pWS630	2μ *URA3* P_{PGK}-Ptr(S117A/H118A/N119A)–HA	This study
pWS631	2μ *URA3* P_{PGK}-Ptr(S196A/K197A/F198A/S199A)–HA	This study
pWS632	2μ *URA3* P_{PGK}-Ptr(R792A/T793A/E794A/E795A)–HA	This study
pWS634	2μ *URA3* P_{PGK}-Ptr(N317A/R318A/S319A)–HA	This study
pWS635	2μ *URA3* P_{PGK}-Ptr(E91A)–HA	This study
pWS638	2μ *URA3* P_{PGK}-Ptr(T127A)–HA	This study
pWS639	2μ *URA3* P_{PGK}-Ptr(T322A/L323A)–HA	This study
pWS647	2μ *URA3* P_{PGK}-Ptr(D79A)–HA	This study
pWS648	2μ *URA3* P_{PGK}-Ptr(F198A)–HA	This study
pWS649	2μ *URA3* P_{PGK}-Ptr(H193A)–HA	This study
pWS656	2μ *URA3* P_{PGK}-Ptr(R792A)–HA	This study
pWS657	2μ *URA3* P_{PGK}-Ptr(Y799A)–HA	This study
pWS663	2μ *URA3* P_{PGK}-PtrC-terminal(S561–E962)–HA	This study
pWS664	2μ *URA3* P_{PGK}-PtrN-terminal(M1–L515)–HA	This study
pWS665	2μ *TRP1* P_{PGK}-PtrC-terminal(S561–E962)–HA	This study
pWS697	2μ *URA3* P_{PGK}-Ptr(S117A)–HA	This study
pWS698	2μ *URA3* P_{PGK}-Ptr(N119A)–HA	This study
pWS699	2μ *URA3* P_{PGK}-Ptr(K197A)–HA	This study

α- and β-subunits. In both subfamilies, where structures have been determined, each individual domain/subunit contains two similarly folded subdomains comprised of a β-sheet and two α-helical bundles. The structural symmetry apparent in the M16 enzyme family is proposed to have arisen as the result of ancient gene duplication events [2].

The non-essential yeast proteins Axl1p and Ste23p can each promote a proteolytic cleavage associated with the production of the **a**-factor mating pheromone [17,18]. This signalling molecule is one of two, the other being α-factor, that are required for the fusion of haploid yeast cells with the opposite mating type (i.e. *MAT***a** and *MAT*α). However, Axl1p and Ste23p should not be considered as being functionally redundant. Axl1p is significantly more efficient at promoting **a**-factor production despite being expressed at a considerably lower level compared with Ste23p [4]. Moreover, Axl1p also has a unique non-proteolytic function in establishing cell polarity in yeast (i.e. bud site selection) [17,19]. Other than a sterile mating phenotype and a budding defect, no additional phenotypes have been reported for an Axl1p and Ste23p double knockout strain.

Despite recent interest in the M16A subfamily, sparked in part by the proposed role of IDE in protecting against Alzheimer's disease, much remains to be determined regarding the physiological roles and enzymatic properties of these proteases. The lack of efficient and quantitative assays for M16A enzyme activity has hindered their functional characterization. Our recent development of yeast as a tractable genetic system for analysing IDE function has prompted us to investigate whether the same system could be developed for functional studies of Ptr [4]. This system relies on the ability of a heterologously expressed M16A enzyme to substitute for the activities of the yeast M16A enzymes Axl1p and Ste23p in **a**-factor pheromone production.

In the present report, we show the successful application of the yeast system for structure–function studies of Ptr. We demonstrate that several motifs with highly conserved primary sequences, in addition to the extended zinc-coordinating HXXEH-motif, are required for the enzymatic function of Ptr and perhaps other M16A enzymes. Our results further suggest that M16A enzymes from mammalian, yeast, and bacterial sources have similar substrate specificity.

EXPERIMENTAL

Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Tables 1 and 2 respectively. Yeast strains were routinely grown at 30 °C on YEPD (yeast extract peptone dextrose) or SC (synthetic complete) dropout medium when propagating plasmid-transformed strains [20]. Yeast transformations were carried out as described previously [21].

pCDK35, pRS316, pSM463, pSM703, pWS371, pWS482 and pWS496 have been described previously [4,22,23]. pWS603 was created by PCR-directed plasmid-based recombination cloning [24]. In brief, the entire *ptr* gene was amplified by PCR using pCDK35 as a template, such that the PCR product had 39 base pair extensions that were homologous to the pSM703 vector into which the gene was recombined. The reverse PCR oligonucleotide was engineered to contain a BglII restriction site before the stop codon of the *ptr* gene. The PCR product was co-transformed into yeast along with pSM703 that had been linearized with BamHI and NotI restriction enzymes. Following homologous recombination and selection on SC − ura solid medium, individual yeast colonies were screened for those containing a plasmid encoding the *ptr* gene (pWS603), as determined by restriction enzyme mapping and subsequent sequencing of the isolated plasmids. A DNA fragment from BglII-digested pWS496 that encodes a triple repeat HA (haemagglutinin)-tag was introduced into the BglII site of pWS603 to create pWS604. Site-directed mutants of Ptr were created by recombination cloning using pWS604 as the recipient vector and a PCR fragment generated with mutagenic PCR oligonucleotides that amplified the appropriate fragment of Ptr for recombination. In these instances, the mutagenic PCR oligonucleotides contained the desired mutation along with a silent restriction site to facilitate identification of the desired plasmid clone; some oligonucleotides were designed to effect loss of a native restriction site. Candidate clones were screened by restriction enzyme digests to identify those with the desired mutation, which were confirmed by DNA sequencing. pWS617 and pWS618 were created by gapping pWS482 and pWS371 respectively with BglII and re-ligating the vector. The parent vectors

each had two tandem copies of a triple repeat HA-tag and subsequent gapping and re-ligation yielded a single triple repeat HA-tag.

Preparation of total cell extracts

Crude protein extracts for Western blot analysis were obtained by NaOH/trichloroacetic acid precipitation [25]. In brief, equal quantities of mid-log cells (an equivalent of 2 ml of culture with a D_{600} of 1.0) were harvested by centrifugation at 16 100 ***g*** for 1 min, rinsed with cold water, resuspended in 1 ml of cold deionized water and treated with 0.24 M NaOH and 0.14 M 2-mercaptoethanol for 15 min on ice. Proteins were precipitated from solution using 11.5 % (w/v) trichloroacetic acid and recovered by centrifugation at 16000 ***g*** for 15 min. Recovered proteins were resuspended in preheated (100 °C) urea sample buffer (250 mM Tris, pH 8.0, 6 M urea, 4 % (w/v) SDS and 0.01 % Bromophenol Blue). Equivalent amounts of samples were evaluated by SDS/PAGE (10 % gels) and Western blot analysis. HA-tagged proteins were probed with mouse anti-HA antibody and horseradish peroxidase-conjugated rabbit anti-mouse secondary antibodies, and detected by enhanced chemiluminescence (BM Chemiluminescence Blotting Substrate; Roche).

Serial dilution yeast mating assay

The ability of Ptr and other M16A enzymes to rescue the mating defect of Y272 (*MAT***a** *axl1Δ ste23Δ*) was determined in accordance with our recently established methods [26]. In brief, plasmid transformed strains were cultured in selective medium for 24 h. The cultures were prepared for the assay by first normalizing the culture cell density to a D_{600} of 1.0 with sterile selective medium. A portion of each normalized *MAT***a** culture was diluted 10-fold with *MATα lys1* cells (strain IH1793) that had been cultured and normalized in parallel in non-selective medium (YEPD), such that the final volume of the mixture was 100 μl. This primary mixture was subjected to several additional 10-fold dilutions with normalized *MATα lys1* cells. A portion of each serially diluted sample (5 μl) was spotted onto SD (synthetic dextrose) solid medium. Growth of diploid cells was scored after 72 h at 30 °C. To verify that the input of *MAT***a** cells was the same for each sample, an additional portion (5 μl) of each diluted sample was spotted onto SC − lys plates. Only unmated *MAT***a** cells and diploids that formed during the handling of the samples can grow on this medium.

Halo assay for a-factor production

The presence of secreted **a**-factor was determined using an established bioassay [27]. In this test, a *MATα* yeast strain that is hypersensitive to the presence of **a**-factor was used to quantitatively determine the amount of pheromone in samples. The **a**-factor samples used in this test were recovered from *MAT***a** cultures as described previously [28]. In brief, this method takes advantage of the observation that the **a**-factor mating pheromone is hydrophobic and readily adsorbs to the walls of polypropylene tubes. Strains were cultured for 3 days in polypropylene tubes and cleared of cellular material by washing the tube several times with deionized water. The adsorbed **a**-factor was extracted from the washed tubes by rinsing the tube walls with methanol and concentrating the rinse by vacuum centrifugation. All samples were normalized to the same final volume using methanol, which was 0.33 % of the original culture volume. Serial 2-fold dilutions of each sample were prepared in YEPD. A portion of each dilution (2 μl) was spotted onto a lawn of *MATα lys1 sst2-1* (strain RC757) cells that had been spread as a thin layer onto a YEPD-agar plate. These cells undergo a strong growth arrest phenotype upon exposure to the **a**-factor mating pheromone. The effect of the sample on *MATα* cell growth was recorded after 24 h at 30 °C. Because the minimum amount of **a**-factor that can elicit a response in this assay is a known constant (12 pg/μl; [29]), the results yielded from the halo assay allowed the measurement of the concentration of **a**-factor in the original sample.

Mass spectroscopy

Secreted **a**-factor was recovered from the walls of polypropylene tubes by organic extraction as described previously [4] and analysed by MALDI–TOF-MS (matrix-assisted laser-desorption ionization–time-of-flight MS). In brief, the **a**-factor-enriched sample was resuspended in 0.1 % trifluoroacetic acid and adsorbed onto a Zip Tip® (C_{18} beads; Millipore). The sample was desalted by washing the Zip Tip® three times with 0.1 % trifluoroacetic acid (10 μl/wash) and the remaining bound material was eluted by 10 μl of 70 % (v/v) acetonitrile in aqueous solution. The concentrated, desalted samples were analysed by MALDI–TOF-MS using an α-cyano-4-hydroxycinnamic acid matrix and a Voyager-DE Biospectrometry™ Workstation (Applied Biosystems).

Invasive growth assay

The ability of haploid yeast cells to invade yeast agar was determined using a plate-washing assay [30]. In brief, cells were cultured in SC − ura and cell suspensions (10^6 cells/ml) were spotted onto either SC − ura- or YEPD-agar plates (5 μl/spot). After 4 days growth at 30 °C, the plates were washed under running H_2O while gently rubbing the surface of the agar plate with a gloved finger to remove surface cells. Agar plates were scanned prior to and immediately after washing. The results associated with the YEPD plates are presented in Figure 7, as the observed phenotype was more robust.

RESULTS

Ptr can be heterologously expressed in yeast

*MAT***a** yeast lacking the native M16A proteases Axl1p and Ste23p (Y272; *MAT***a** *axl1Δ ste23Δ*) are unable to mate due to an inability to produce mature **a**-factor. In this strain, **a**-factor production, and consequently yeast mating, can be restored by plasmid-based expression of either of the yeast M16A enzymes or heterologous expression of rat IDE, a highly homologous member of the M16A subfamily [4]. This observation suggests that M16A enzymes may have conserved substrate specificity. To expand on this hypothesis, we have tested the ability of prokaryotic Ptr to substitute for the activity of the yeast enzymes *in vivo*.

To test our hypothesis, we first generated a yeast expression plasmid encoding Ptr–HA (HA epitope-tagged Ptr) under control of the strong constitutive phosphoglycerate kinase promoter. The HA-tag was fused to the 3′ end of the *ptr* gene. Placement of an epitope-tag at this position was previously shown not to interfere with the activities of other M16A proteases [4]. A whole cell extract prepared from Y272 (*MAT***a** *axl1Δ ste23Δ*) cells expressing Ptr–HA contained two immunoreactive bands with sizes of approx. 104 and 109 kDa. The slower migrating band has a mass consistent with that predicted for Ptr–HA (108.8 kDa, upper band; Figure 1), while the faster migrating band is likely to represent

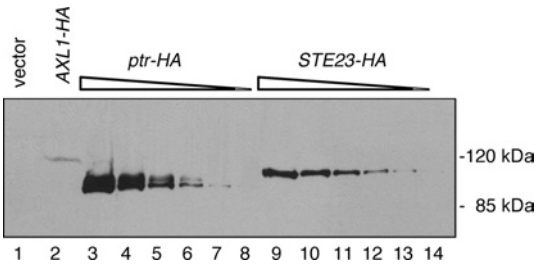

Figure 1 Ptr–HA expressed in yeast migrates at its expected molecular mass

Protein extracts were prepared from yeast containing an empty vector, or vectors encoding C-terminally HA-tagged Axl1p, Ptr, or Ste23p. Equivalent amounts of total protein extracts for each sample (lanes 1–3 and 9) and 2-fold serial dilutions of Ptr (lanes 4–8) and Ste23p (lanes 10–14) samples were analysed by SDS/PAGE and immunoblot using an anti-HA antibody. Ptr was routinely detected as a doublet. The strains used were Y272 transformed with pRS316 (empty vector), pWS604 (Ptr–HA), pWS617 (Ste23p–HA) and pWS618 (Axl1p–HA).

an N-terminal truncated product, or a product derived from an alternative start codon (e.g. Met53). Extracts derived from yeast expressing Axl1p–HA (HA-epitope tagged Axl1p) and Ste23p–HA (HA-epitope tagged Ste23p) were also evaluated for comparison with Ptr–HA (Figure 1).

Heterologous expression of Ptr can promote yeast mating

To evaluate whether heterologously expressed Ptr–HA could substitute for the yeast M16A enzymes in promoting yeast mating, a process that requires the **a**-factor mating pheromone, we determined if Ptr expression could rescue the mating defect of Y272 (*MAT***a** *axl1*Δ *ste23*Δ). The relative ability of Ptr–HA to promote yeast mating was scored using a serial dilution mating test that has been described previously [26]. We observed that Ptr–HA could indeed promote yeast mating as evidenced by the formation of diploid cells on selective media (Figure 2A). Mating was less efficient by comparison with Axl1p–HA and similar in extent by comparison with Ste23p–HA. In previous studies, rat IDE was shown to have activity that was comparable with Ste23p [4]. An emerging theme from these studies is that M16A enzymes are generally inefficient at promoting **a**-factor production, which holds true even when M16A enzymes are artificially over-expressed relative to Axl1p.

Ptr can produce the mature form of a-factor

Yeast mating depends on a number of factors, including proper production of the mature form of **a**-factor. To verify that the observed differences in mating efficiency correlated with differences in the levels of pheromone production, the **a**-factor secreted from Ptr-expressing cultures was isolated and evaluated using a halo assay. This experiment was carried out to ensure that Ptr was not simply failing to complement for a possible unknown function of Axl1p and Ste23p in promoting the yeast mating process. Evaluation of the **a**-factor samples revealed that Ptr- and Ste23p-expressing strains produced comparable amounts of **a**-factor, as judged by biological activity, and both produced significantly less **a**-factor than an Axl1p-expressing strain (Figure 2B). The simplest explanation for our findings is that Ptr is able to direct pheromone production, albeit not at the same level as Axl1p.

We have demonstrated previously that the decreased activity of Ste23p relative to Axl1p is not simply due to an inability to achieve exact cleavage at the native cleavage site; an **a**-factor molecule shortened by one amino acid has one quarter of the activity of

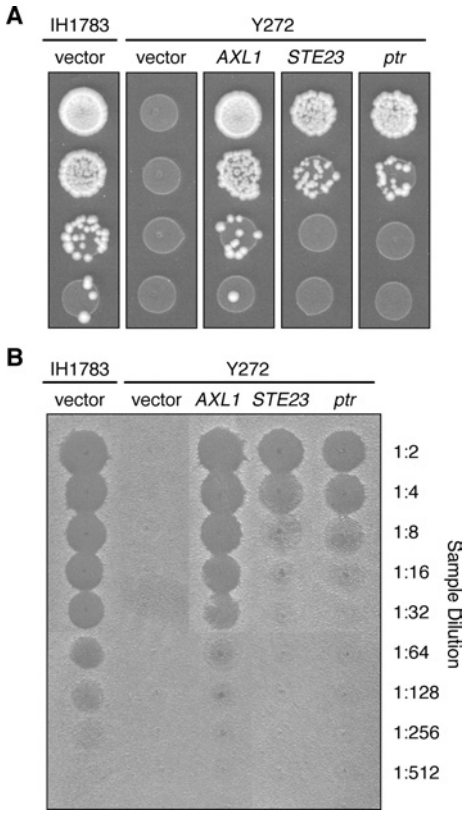

Figure 2 Ptr can promote a-factor production

Two assays were performed to determine the extent of **a**-factor production by Ptr. (**A**) Yeast strain Y272, which lacks the yeast M16A proteases, was transformed with plasmids encoding the indicated M16A proteases and evaluated for its ability to mate using the serial dilution mating assay. A wild type *MAT***a** strain (IH1783) was transformed with an empty vector and evaluated in parallel. In this assay, a fixed number of IH1793 *MAT*α cells (10^5 cells) were mixed with a serially diluted amount of the indicated *MAT***a** cells (10^4, 10^3, 10^2 and 10^1 cells), and the resultant cell mixtures spotted onto an SD minimal plate. The input amount of *MAT***a** cells for the first row is 10^4 cells. The growth of diploids on the selective media indicates that mating has occurred. (**B**) Concentrated **a**-factor samples recovered from the conditioned media of yeast expressing the indicated M16A protease were analysed by the spot halo test. Two-fold serial dilutions of each sample were spotted onto a lawn of RC757 *MAT*α cells, whose growth is arrested in the presence of pheromone. Formation of a spot in the lawn of cells is indicative of the presence of **a**-factor. The highest dilution with activity is considered the endpoint and is equivalent to 12 pg/μl of **a**-factor. The *MAT***a** strains used were IH1783 and Y272, which were transformed with pRS316 (empty vector), pWS618 (Axl1p–HA), pWS617 (Ste23p–HA), and pWS604 (Ptr–HA).

full-length pheromone [4,31]. Similarly, we have determined that Ptr can also produce full-length mating pheromone, as confirmed by MS analysis. When conditioned medium used for growth of a Ptr-expressing yeast strain was enriched for **a**-factor, and then analysed, a species of 1629 Da was detected (Figure 3A; block arrow). This 1629 Da species is the most prominent species observed within such samples and corresponds with the predicted molecular mass of the mature **a**-factor mating pheromone. A 1629 Da species is also observed in a sample derived from an Axl1p-expressing strain (Figure 3B), but is absent in a strain lacking M16A enzymes (Figures 3C). No significant peaks are detected in the Ptr-derived sample at molecular masses that would correspond to **a**-factor species that are one amino acid smaller or larger than the full-length molecule. The additional peaks observed in the Ptr and Axl1p samples (Figure 3, open arrows) do not correspond to any known **a**-factor species and were not reproducible between experiments.

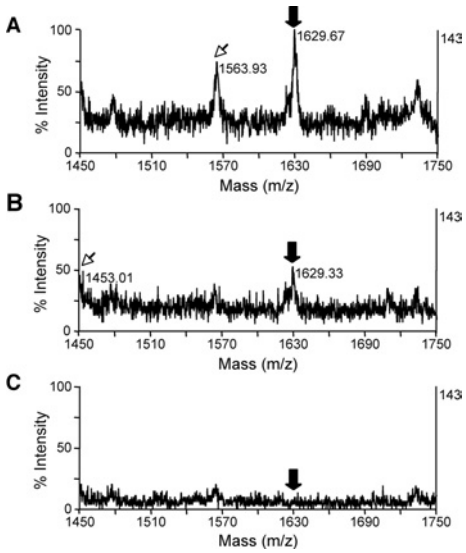

Figure 3 Mass spectroscopic analysis of a-factor produced by Ptr

*MAT***a** yeast expressing Ptr or Axl1p, but otherwise deficient for the yeast M16A proteases, were grown in selective media, and secreted **a**-factor was enriched as described in Experimental procedures. Samples enriched for **a**-factor were subjected to MALDI–TOF-MS using an α-cyano-4-hydroxycinnamic acid matrix. The strains used were Y272 transformed with (**A**) pWS604 (Ptr–HA), (**B**) pWS618 (Axl1p–HA) or (**C**) pRS316 (empty vector). All strains also contained pSM463 (*2μ TRP1 MFA1*). Block arrows indicate the position of the 1629 Da peak expected for **a**-factor; open arrows indicate the position of additional peaks that were neither reproducibly detected nor correlated with any known **a**-factor species.

Multiple motifs that are conserved among M16A enzymes are required for Ptr function

To date, most mutational studies of the M16A subfamily have focused on residues that comprise the extended metalloprotease motif ($HXXEHX_{69}EX_6E$). For example, the only known inactivating mutations of Ptr are within this motif [32]. Besides the extended metalloprotease motif (motif Ia), several additional regions of highly conserved primary sequence can be identified among M16A enzymes [4] and are shown in Figures 4(A), 5(A) and 5(B). The importance of these motifs to the function of Ptr, or any other M16A enzyme, has not yet been evaluated. Using the genetic system that we have developed as a measure of M16A function and with Ptr as the model M16A enzyme, we have tested the hypothesis that these motifs are functionally important for the proteolytic function of Ptr.

One prediction of our hypothesis is that the mutation of residues within conserved motifs should compromise the function of yeast expressed Ptr. To test this prediction, we initially mutated residues within motif Ia and evaluated the effects on activity using the yeast mating assay. Within motif Ia, Glu^{91} of the extended metalloprotease motif was mutated to alanine (E91A), as the Glu residue is invariably conserved among M16A proteases. Although E91A is not thought to be involved in the coordination of the catalytic zinc ion, it is essential for the proteolytic function of Ptr [3]. The same is true for the equivalent glutamate in other M16A subfamily enzymes [3,4,17]. As expected, Ptr E91A exhibited no proteolytic activity, which was shown by a lack of yeast mating (Figure 4B). The lack of activity cannot be readily explained by a lack of protein expression since the mutant is expressed as well as the wild-type enzyme, as judged by Western blot analysis (results not shown). Conversely, Asp^{79}, which is also invariably conserved within motif Ia, was not required for proteolytic activity.

Several novel multiple- and single-site alanine substitution mutants of Ptr–HA, hereafter referred to as MSMs (multiple-site mutations) and SSMs (single-site mutations) were created at other conserved regions and evaluated for functional significance. Mutations of motif Ib (MSM, S117A/H118A/N119A; SSM, N119A) and motif Ic (MSM S196A/K197A/F198A/S199A), which are both located within the N-terminal half of Ptr, disabled the ability of the enzyme to promote yeast mating (Figure 4B). The mutants were expressed at levels comparable with the wild-type enzyme (results not shown). Not all mutations within these motifs were inactivating. Certain SSMs of conserved residues within each of these regions (S117A, T127A, H193A, K197A, and F198A) remained functional (Figure 4B and results not shown). Mutations of motif II (MSMs N317A/R318A/S319A and T322A/L323A), also within the N-terminal region of Ptr,

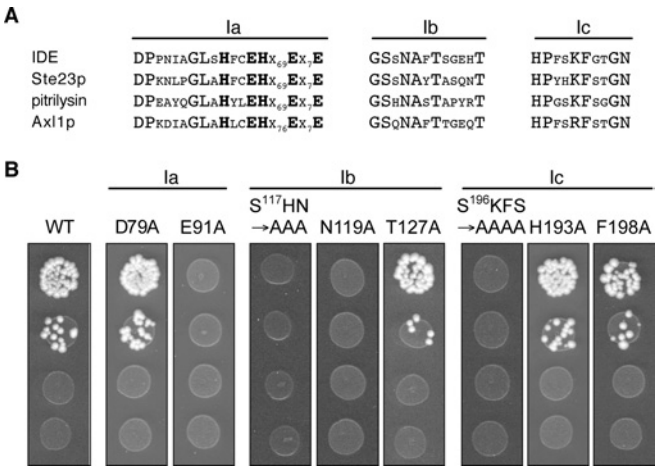

Figure 4 Novel non-functional mutants of Ptr

(**A**) M16A subfamily enzymes have conserved regions throughout the length of their sequences. Three regions of significant similarity ($\geqslant$50% identity spanning $\geqslant$10 amino acids) can be found within the first 300 amino acids of these M16A proteases. These regions have been termed motifs Ia, Ib and Ic. Conserved residues are shown in a larger font. The residues of the extended metalloprotease motif are in bold. (**B**) A strain lacking the yeast M16A proteases (Y272) was transformed with a plasmid encoding the indicated Ptr mutant and evaluated using the serial-dilution mating assay as described in the Experimental section and Figure 2. WT, wild-type

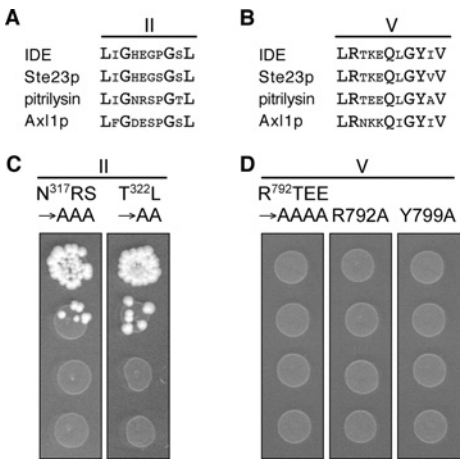

Figure 5 Mutational analysis of motifs II and V of Ptr

(**A** and **B**) Two additional regions of significant similarity, motifs II and V, lie in the N-terminal and C-terminal halves of Ptr respectively. (**C** and **D**) A strain lacking the yeast M16A proteases (Y272) was transformed with a plasmid encoding the indicated motif II (**C**) or motif V (**D**) mutant of Ptr and the strain was evaluated using the serial-dilution mating assay as described in the Experimental section and Figure 2.

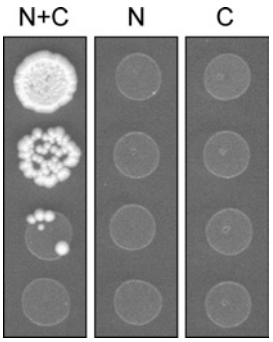

Figure 6 Both structural domains of Ptr are required for activity

The structure of Ptr reveals two independently folded domains that are about equal in mass. When both domains are co-expressed (N- and C-terminal) in a strain lacking the yeast M16A proteases (Y272), mating is observed as judged by the serial-dilution mating assay described in the Experimental section and Figure 2. Neither the N-terminal (N) nor C-terminal domain (C) alone can promote mating.

similarly had no effect on function (Figure 5C). In part, MSM N317A/R318A/S319A was chosen to confirm that mutations of non-conserved residues within highly conserved motifs were not inactivating. By contrast, mutations of motif V, which lies in the C-terminal half of Ptr (MSM R792A/T793A/E794A/E795A and SSMs R792A and Y799A), were all inactivating (Figure 5D). Motif V has previously been postulated to play a role in the function of M16A subfamily enzymes [5]. As with other inactive Ptr–HA mutants, the expression of motif V mutants was not adversely affected (results not shown).

Both N- and C-terminal domains of Ptr are essential for promoting yeast mating

Structural analysis of Ptr reveals that the enzyme adopts a modular conformation. The enzyme has two domains of approximately equal mass (approx. 50 kDa), herein referred to as N- and C-terminal domains, that are positioned about what appears to be a central cavity of approx. 25–35 Å in diameter. These N- and C-terminal domains are connected by a linker region of approx. 20 amino acids that lacks ordered secondary structure and may have some flexibility.

The role of the Ptr C-terminal domain, which lacks the zinc-coordinating active site, is currently unknown. Our mutational analysis suggests that this domain contains at least one highly conserved region that is important for enzyme function (motif V; see Figure 5D). However, we could not prematurely exclude the possibility that inactivating mutations of this C-terminal motif might be altering the structure of the full-length enzyme so as to render the catalytic site within the N-terminal domain inaccessible to **a**-factor. To address this issue, we independently expressed the N- (residues Met^{1}–Lys^{515}) and C-terminal (residues Ser^{516}–Glu^{962}) domains of Ptr in Y272 (*MAT***a** *axl1Δ ste23Δ*) and evaluated yeast mating. Neither domain alone was capable of promoting yeast mating, suggesting that catalytic activity is not intrinsic to the N-terminal domain alone (Figure 6). Co-expression of both domains restored yeast mating. Taken together, we interpret these results to mean that the N- and C- terminal domains of Ptr function in a cooperative manner for the enzyme's proteolytic function. Further, the N- and C-terminal domains of other M16A subfamily enzymes may have a similar complementary function. Indeed, we have observed using the same system that IDE also requires co-expression of both of its domains for activity (results not shown).

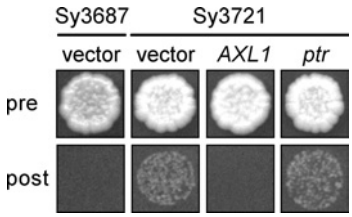

Figure 7 Ptr cannot repress haploid invasive growth

Plasmids encoding HA-tagged Ptr and Axl1p were transformed into an *axl1* haploid strain (Sy3721). Equal cell density suspensions of these strains were spotted onto SC − ura and grown for 3 days. The plates were digitally scanned before (pre) and after (post) washing with running water to remove surface lying cells. The presence of cells after washing indicates invasive growth. Strains used were Sy3687 transformed with pRS316 (empty vector) and Sy3721 (*axl1::HIS3*) transformed with pRS316 (empty vector), pWS618 (Axl1p–HA) and pWS604 (Ptr–HA).

Ptr is not fully redundant with Axl1p

Axl1p is a multi-functional enzyme [17]. Besides being required for **a**-factor production, Axl1p is required for maintenance of the axial budding pattern that is characteristic of haploid yeast and for repressing their invasive growth [30,33]. These two latter functions require an undefined non-proteolytic activity of Axl1p. To address the extent of functional conservation between Ptr and Axl1p, we heterologously expressed Ptr in an Axl1p-deficient strain (Sy3721) that normally displays hyperinvasive growth and performed an invasion growth test (Figure 7). Our results indicated that Ptr could not repress haploid invasive growth. When this result is considered with observations that neither IDE nor Ste23p can repress invasive growth, it suggests that Axl1p has adopted a specialized role for this purpose. Moreover, the relatively limited ability of Ste23p, IDE and Ptr to promote **a**-factor maturation by comparison with Axl1p further supports a specialized role for Axl1p in pheromone biosynthesis.

DISCUSSION

This report describes the first genetically tractable system for functional studies of Ptr. We have demonstrated that Ptr can be expressed in *Saccharomyces cerevisiae* and that the heterologously expressed enzyme can utilize the yeast **a**-factor precursor as a surrogate substrate (Figures 1 and 2). Our observations suggest that the yeast-associated phenotype can be used to better understand structure–function relationships in Ptr, despite the fact that native substrates of Ptr are unknown and the deletion of Ptr results in no readily observable phenotype in *E. coli* [32,34]. Moreover, the use of a single system for functional studies of M16A enzymes provides a means to compare, contrast and test new hypotheses related to the enzymatic properties of these proteins.

With respect to structure–function relationships, we have confirmed the essential requirement for motif Ia of Ptr in enzymatic function (Figure 4). In addition, we have demonstrated that several motifs conserved among M16A enzymes (motifs Ib, Ic and V) are also essential for Ptr activity (Figures 4 and 5). These results represent the first experimental evidence that motifs outside motif Ia are in fact required for the proteolytic activity of Ptr and may prove useful in directing future studies of the M16A subfamily. The essential nature of Asn^{119} within motif Ib can be somewhat rationalized on the basis of the known structure of Ptr. This residue is not part of the extended metalloprotease motif but is positioned proximal to the catalytic zinc ion and faces the central pocket of the protease's N-terminal domain. Thus we speculate that mutations of this motif result in structural defects that either impact

on zinc coordination or on the overall folding of the N-terminal domain active site. The essential nature of motif Ic, which is considerably distal to the catalytic zinc ion, is more difficult to rationalize. It is conceivable that the mutations created within motif Ic result in conformational defects that affect enzyme function. This is consistent with our observation that only MSMs and not SSMs generated within this motif had an impact on enzyme function. The exact role of motif V, which is located within the C-terminal domain of M16A subfamily enzymes, also remains to be elucidated. In Ptr, this motif is positioned at the entrance of the central cavity in close spatial proximity to the catalytic zinc ion associated with the N-terminal domain. Molecular threading predicts a Ptr-like structure for IDE, Ste23p and Axl1p, and a similar orientation for motif V within each of these enzymes. On the whole, the structure of Ptr and other M16A enzymes is strikingly similar to that of the MPP, an M16B family member [35]. Structural analyses of MPP have revealed the presence of a C-terminal glycine-rich loop within the α-subunit that restricts access to the enzyme's catalytic zinc ion, which is associated with the β-subunit. This loop, which contributes significantly to MPP substrate specificity, appears structurally analogous to motif V of Ptr [36]. Thus it is tempting to speculate that this region may contribute to the substrate specificity of Ptr and other M16A enzymes. Additional studies will be required to confirm whether this is indeed the case. It should be stressed, however, that we cannot discount alterations in protein localization, in addition to improper protein folding, as the underlying cause of functional defects associated with the inactivating mutations reported in this study.

The exact role of conserved motif II is somewhat more enigmatic. Our limited mutational analysis of motif II suggests that, unlike motifs Ia, Ib Ic and V, it is not essential for Ptr function. Consistent with this observation, we note that motif II is not spatially positioned near the catalytic zinc ion or other essential motifs, suggesting an alternative role for this highly conserved region. Still, it remains possible that motif II mutations have more subtle effects, which are being overlooked by our assays. The genetic assay used in this study is highly sensitive, but it cannot easily discriminate between wild-type activity and that of mutants having greater than 30 % activity [26]. If additional mutations fail to have an observable impact on Ptr activity, more quantitative measures of yeast mating and/or *in vitro* approaches may be needed to determine whether motif II has an impact on Ptr activity. Conversely, motif II may simply have no impact on enzyme function.

We have also demonstrated that the N-terminal domain of Ptr alone does not possess enzymatic activity, which is consistent with our observation that a C-terminal domain motif is essential for Ptr function. We do not believe that the absence of activity reflects a lack of assay sensitivity, since Axl1p is also inactive as a half molecule in our genetic tests (results not shown). The simplest explanation for our observation is that cooperativity exists between the N- and C-terminal domains of Ptr. Whether this cooperativity involves the direct interaction of the two domains to form the equivalent of a full-length enzyme, or interactions that stabilize a proper quaternary structure that is necessary for activity remains to be determined. Interestingly, MPP also requires both of its domains for enzymatic activity and efficient substrate binding [37–40]. These observations, when combined with our own, suggest that cooperativity between the two domains of M16 family proteases is a general feature of this class of enzymes. Indeed, we have observed a similar cooperative requirement for IDE using our genetic system (results not shown). However, it should be noted that a recent report indicates that IDE is partly functional in the absence of a large portion of its C-terminal domain [41]. The truncated enzyme is reported to have only 2 % of wild-type activity and has altered substrate specificity, being able to cleave only one of two IDE substrates evaluated in the study [41].

With respect to the substrate specificity of Ptr and M16A enzymes on the whole, we can make some generalizations. Our observations support a conserved role for the M16A subfamily in the general proteolysis of small peptide molecules. Indeed, it is well-documented that certain M16A enzymes, such as IDE and Ptr, have fairly broad substrate specificity. We hypothesize that Ste23p is also likely to have broad substrate specificity because of its high degree of similarity to IDE and Ptr, but ultimate validation of this hypothesis will require the development of an *in vitro* assay for Ste23p. It should be noted that we have not yet been able to demonstrate that Ste23p can cleave insulin [4]. The relatively higher activity of Axl1p in promoting pheromone production suggests that it may have adopted a narrow substrate specificity, allowing for its specialization in the biosynthesis of **a**-factor. Further supporting a specialized role for Axl1p is the apparent inability of other M16A enzymes to promote the Axl1p-dependent events of axial budding and the repression of haploid invasive growth. Consistent with its proposed specialized role, Axl1p is the most divergent of the four enzymes. The functional role of the other M16A enzymes remains undetermined, but we speculate, as others have, that these enzymes cleave oligopeptides that are cytotoxic, amyloidogenic, or otherwise not easily degraded [9,42].

Our results also support the proposed structure-based recognition of substrates by M16A enzymes. A common property of M16A substrates is a predicted or established ability to adopt a common secondary structure, a β turn [43,44]. Although the yeast P2 **a**-factor intermediate shares no sequence homology with other M16A substrates (e.g. Aβ peptide and insulin), the mature portion of the pheromone is predicted to contain a Type-II β-turn [45–47]. The observation that four M16A enzymes can recognize the P2 **a**-factor intermediate suggests that resolving its structure may yield significant insight into the enzymatic properties of these enzymes.

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