Published in final edited form as:

Yeast. 2009 November ; 26(11): 595–610. doi:10.1002/yea.1709.

Yeast Ste23p shares functional similarities with mammalian insulin-degrading enzymes

Benjamin J. Alper, Jarrad W. Rowse, and Walter K. Schmidt*

Department of Biochemistry and Molecular Biology, University of Georgia, Athens, GA 30602, USA

Abstract

The *S. cerevisiae* genome encodes two M16A enzymes: Axl1p and Ste23p. Of the two, Ste23p shares significantly higher sequence identity with M16A enzymes from other species, including mammalian insulin-degrading enzymes (IDEs). In this study, recombinant Ste23p and *R. norvegicus* IDE (*RnIDE*) were isolated from *E. coli*, and their enzymatic properties compared. Ste23p was found to cleave established *RnIDE* substrates, including the amyloid- β peptide (A β 1–40) and insulin B-chain. A novel internally quenched fluorogenic substrate (Abz–SEKKDNYIIKGV–nitroY–OH) based on the polypeptide sequence of the yeast P2 a-factor mating propheromone was determined to be a suitable substrate for both Ste23p and *RnIDE*, and was used to conduct comparative enzymological studies. Both enzymes were most active at 37 °C, in alkaline buffers and in high salt environments. In addition, the proteolytic activities of both enzymes towards the fluorogenic substrate were inhibited by metal chelators, thiol modifiers, inhibitors of cysteine protease activity and insulin. Characteristics of *STE23* expression were also evaluated. Our analysis indicates that the 5' terminus of the *STE23* gene has been mischaracterized, with the physiologically relevant initiator corresponding to residue M53 of the publicly annotated protein sequence. Finally, we demonstrate that, unlike haploid-specific Axl1p, Ste23p is expressed in both haploid and diploid cell types. Our study presents the first comprehensive biochemical analysis of a yeast M16A enzyme, and provides evidence that *S. cerevisiae* Ste23p has enzymatic properties that are highly consistent with mammalian IDEs and other M16A enzymes.

Keywords

yeast; Ste23p; insulin-degrading enzyme; M16A; metalloprotease; Alzheimer's disease

Introduction

The M16A subfamily comprises a group of large (~100 kDa), zinc-dependent metallo-endopeptidases, which are found within all prokaryotic and eukaryotic organisms that have been thoroughly examined (Rawlings, 2008). M16A enzymes are characterized by the presence of an essential HxxEH motif, generally located within 200 residues of the amino-

*Correspondence to: Walter K. Schmidt, A416 Life Sciences Building, Athens, GA 30602, USA. wschmidt@bmb.uga.edu.

terminus. These residues are responsible for zinc coordination and catalysis (Becker and Roth, 1992).

Structural characterization of M16A subfamily enzymes has been reported (Im *et al.*, 2007; Maskos, 2005; Shen *et al.*, 2006). M16A enzymes have a quaternary structure that is similar to a clamshell in appearance, with N- and C-terminal bowl-shaped domains of approximately 50 kDa connected by a short linker region of 20–30 residues. Both the N- and C-terminal domains, as well as individual residues from highly conserved sequences within each domain, are required for the catalytic function of M16A enzymes (Alper *et al.*, 2006; Becker and Roth, 1993; Ding *et al.*, 1992; Kim *et al.*, 2005; Li *et al.*, 2006; Perlman *et al.*, 1993). Recent studies of the human insulin-degrading enzyme (*HsIDE*) and bacterial pitrilysin (*EcPtr*) have demonstrated that M16A enzymes can adopt either an ‘open’ conformation, which is hypothesized to permit substrate access to a central catalytic compartment, or a ‘closed’ conformation, which is presumed to be necessary for proteolysis, yet likely prohibits entrance and exit of substrates from the catalytic site (Maskos, 2005; Shen *et al.*, 2006). Transitions between the ‘open’ and ‘closed’ conformations of M16A enzymes are hypothesized to represent a rate-limiting step within these enzymes’ catalytic cycles. Consistent with this hypothesis, mutations that disrupt interactions between the N- and C-terminal domains of *HsIDE* increase catalytic activity by as much as 40-fold, presumably by facilitating the transition between the open and closed conformations of the enzyme (Shen *et al.*, 2006). Allosteric regulation of *HsIDE* and *Rattus norvegicus* IDE (*RnIDE*) by ATP has been reported, although the underlying reason for such regulation is not understood at present (Camberos, 2001; Song *et al.*, 2004). Eukaryotic M16A enzymes also demonstrate sensitivity to N-ethylmaleimide (NEM) and other thiol modifiers, although the mechanistic importance of cysteine residues within these enzymes remains poorly elucidated (Becker and Roth, 1995; Kim *et al.*, 2005; Neant-Fery *et al.*, 2008).

M16A enzymes have broad yet selective substrate specificity. Established substrates of M16A enzymes include insulin, amyloid- β (A β) peptides, glucagon, amylin, plant systemin and yeast P2 pro-a-factor, among others (Adames *et al.*, 1995; Alper *et al.*, 2006; Bennett *et al.*, 2000; Huet *et al.*, 2008; Kim *et al.*, 2005; Kirschner and Goldberg, 1983; Qiu *et al.*, 1998). Structural studies of *HsIDE* in complex with multiple peptide substrates have revealed several features that play a role in substrate recognition by this and presumably other M16A enzymes (Maskos, 2005; Shen *et al.*, 2006). To be recognized by *HsIDE*, potential substrates must be capable of anchoring within the enzyme’s catalytic binding pocket and forming favourable interactions with enzymatic β -strands proximal to the site of peptide hydrolysis. Potential substrates must also be small enough (~50 amino acids in length) to permit encapsulation within the central chamber of the closed enzyme. Peptides with significant positive charge at their C-termini are generally poor substrates, due to unfavourable electrostatic interactions with the positively charged face of the central chamber contributed by the C-terminal domain of the enzyme (Shen *et al.*, 2006). No absolute primary sequence determinant has been reported among substrates of the M16A subfamily. However, studies of *RnIDE* using synthetic fluorogenic peptides have shown that the enzyme preferentially cleaves on the amino side of hydrophobic and basic residues (Song *et al.*, 2005), and studies of *S. lycopersicum* IDE (*SlIDE*) using various alternative

peptide substrates have further suggested a limited preference for proline one or two residues N-terminal to the site of proteolytic cleavage (Huet *et al.*, 2008), which may reflect an overall preference for substrates that adopt a β -turn structure (Kurochkin, 1998).

Medical interest in the M16A subfamily has been motivated in part by the proposal that *HsIDE* serves a protective function in forestalling the onset of Alzheimer's disease (AD) (Hardy and Selkoe, 2002; Selkoe, 2001). Human population studies have indicated that variations in and around the chromosomal locus encoding *HsIDE* are risk factors for AD and type II diabetes mellitus (DM2) (Ertekin-Taner *et al.*, 2000; Groves *et al.*, 2003; Kim *et al.*, 2007; Myers *et al.*, 2000). Consistent with these observations, IDE deficiency or loss of function in rodent models has been correlated with increased levels of A β and insulin, as well as increased incidence of AD and DM2 (Farris *et al.*, 2003; Leissring *et al.*, 2003; Miller *et al.*, 2003). Levels of soluble and insoluble A β and premature death rates are also significantly reduced in transgenic rodent models in which both IDE and the amyloid precursor protein (APP) have been overexpressed (Leissring *et al.*, 2003).

Ste23p and Axl1p are the M16A enzymes native to *Saccharomyces cerevisiae*. Genetic studies support involvement of Ste23p and Axl1p in exacting an N-terminal endoproteolytic cleavage of the P2 precursor of the **a**-factor mating pheromone that is produced by *MATa* haploid cells. *MATa* yeast that lack functional copies of both Ste23p and Axl1p cannot signal mating competency to cells of the complementary mating type, and thus exhibit a 'sterile' mating phenotype (Adames *et al.*, 1995). Axl1p expression is restricted to haploid yeast (Fujita *et al.*, 1994), where, in addition to its role in **a**-factor processing, the enzyme has several other known functions. These include maintenance of a haploid-specific axial budding pattern (Adames *et al.*, 1995; Fujita *et al.*, 1994), repression of haploid-specific invasive growth (Cullen and Sprague, 2002; Palecek *et al.*, 2000) and promotion of efficient haploid cell fusion during mating (Elia and Marsh, 1996). As documented in this study, and by contrast to the haploid-specific expression of Axl1p, Ste23p is expressed in both haploid and diploid yeast. Despite its broad expression profile, the only known function of Ste23p is in **a**-factor processing, a *MATa*-specific event. Its role in *MATa* and diploid cells, which may also be conserved in *MATa* cells, remains enigmatic. Ste23p and Axl1p are not functionally equivalent in terms of their ability to promote mature **a**-factor production. Ste23p is responsible for at most 5% of all **a**-factor production, yet it is expressed at steady-state levels that are at least 10 times higher than that observed for Axl1p (Adames *et al.*, 1995; Alper *et al.*, 2006). These observations suggest an alternative biological function of Ste23p that has yet to be identified.

We now report a comparative biochemical analysis of yeast Ste23p and *RnIDE*. We demonstrate that Ste23p exhibits conserved specificity towards established *RnIDE* substrates A β 1–40 and insulin B-chain, and has enzymatic characteristics that are similar to those of *RnIDE* and other eukaryotic M16A enzymes. In addition, we provide genetic and biochemical evidence that the 5' terminus of the *STE23* ORF has been mischaracterized. This study presents the first detailed biochemical analysis of a fungal M16A enzyme, and supports the suitability of Ste23p as a model for continuing studies of the M16A subfamily within *S. cerevisiae*.

Materials and methods

Sequence comparisons

Polypeptide sequences of M16A enzymes from several commonly studied organisms were evaluated using the multiple global sequence alignment function of Clustal W 2.0.10 with default parameters (Larkin *et al.*, 2007; Wilbur and Lipman, 1983). Polypeptide sequences for *HsIDE*, *RnIDE*, *Drosophila melanogaster* IDE (*DmIDE*), *SlIDE* and *EcPtr* are equivalent to the UniProtKB/Swiss-Prot protein sequence entries P14735, P35559, P22817, Q93YG9 and P05458, respectively (Leinonen *et al.*, 2004). The polypeptide sequence for *Caenorhabditis elegans* IDE (*CeIDE*) corresponds to WormBase protein entry WP: CE37861 (Bieri *et al.*, 2007; Stein *et al.*, 2001). Polypeptide sequences for Ste23p and Ax11p correspond to the Ste23p and Ax11p protein sequences annotated within the *Saccharomyces* Genome Database (Cherry *et al.*, 1998). The polypeptide sequence for *Ustilago maydis* Ste23p (*UmSte23p*) was inferred from *U. maydis* genomic locus UM03257.1 (Harvard, 2008), and the polypeptide sequence for *Schizosaccharomyces pombe* (*SpSte23p*) was inferred from *Sz. pombe* genomic locus NP_593966 (Wood *et al.*, 2002). Graphical representation of the Ste23p and *RnIDE* alignment was created using BOXSHADE 3.21.

Yeast strains and plasmids

The yeast strains and plasmids used in this study are listed in Tables 1 and 2, respectively. Yeast strains were grown at 30 °C in solid or liquid rich media (YEPA) or synthetic complete (SC) dropout medium. Plasmids p80, pRS316, pWS371, pWS375, pWS482 have been described previously (Adames *et al.*, 1995; Kim *et al.*, 2005; Sikorski and Hieter, 1989). Plasmids having 5' sequence deletions (pWS759-763) were created by direct subcloning of PCR products derived from pWS375, which were designed to lack the appropriate DNA region and contain *KpnI* and *HindIII* restriction sites that were subsequently used to insert the PCR product into corresponding sites of pWS482; the *KpnI* site is within the pWS482 polylinker, and *HindIII* is internal to the *STE23* ORF. A plasmid lacking 5' sequence and the first predicted 156 nucleotides of *STE23* (pWS765) was created in a similar manner. pWS908 and pWS909 were obtained commercially (Norclone Biotech Laboratories, London, Ontario, Canada). Yeast transformations were carried out according to established methods (Elble, 1992).

Bacterial strains and plasmids

Bacterial strains and plasmids used within this study are included in Tables 1 and 2. DH5 α *Escherichia coli* (Invitrogen, San Diego, CA, USA) was used for amplification of plasmid DNA, and BL21(DE3) *E. coli* (Novagen, Darmstadt, Germany) was used for protein expression. pET30-b⁺ (Novagen) was used as the vector for recombinant expression of Ste23p and *RnIDE*. Bacterial cells were prepared for chemical transformation according to established methods (Hanahan, 1983).

Vectors for recombinant expression of *STE23* and *RnIDE* were created within pET30-b⁺, such that each encoded an N-terminal polyhistidine tag immediately behind a start codon. pWS769 (6 $\times$ His-Ste23_{pM53-E1027}) was created by subcloning a PCR fragment encoding the region of the *STE23* gene corresponding with residues M53-E1027 of the annotated Ste23p

polypeptide sequence. This sequence was amplified from pWS375 such that an *NdeI* site, an ATG codon and sequence encoding a 6×His tag were created at the 5′ end of the fragment and a *NotI* site was created at the 3′ end. This strategy permitted direct subcloning into corresponding sites within the pET-30b⁺ vector. Construction of pWS804 (6×His-*RnIDE*_{M42-L1019}) was achieved in an analogous fashion and has been described (Alper and Schmidt, 2009). DNA sequencing analysis revealed two mutations encoding single-site amino acid substitutions Y248C and E768A within the plasmid encoding *RnIDE*. The corresponding native residues are not conserved between *RnIDE* and Ste23p, and are positioned distal to the catalytic site, as inferred from analogous residues within *HsIDE*, the most closely related *RnIDE* homologue for which structural data is available.

Enzyme purification

6×His-Ste23p_{M53-E1027} and 6×His-*IDE*_{M42-L1019} were expressed within BL21 (DE3) *E. coli* and purified by immobilized nickel affinity chromatography, according to our established methods (Alper and Schmidt, 2009). In brief, a clarified cell lysate containing the protein of interest was applied onto a HisTrapTM Fast Flow 5 ml column that was attached to an AKTApurifierTM fast protein liquid chromatography (FPLC) system (GE Healthcare, Piscataway, NJ, USA). Block elution with 0.5 M imidazole was used to recover the bound protein after washes to remove unbound material. The imidazole concentration in the eluted sample was reduced by first concentrating the sample to approximately 1 ml, using an Amicon UltraTM-15 ml 100 kDa MW cut-off centrifugal filter device (Millipore, Bedford, MA, USA), and then diluting the sample approximately 10-fold with lysis buffer [50 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid (HEPES), 140 mM NaCl, pH 7.4]. The dilution and concentration steps were repeated once more prior to diluting the final sample to 1 mg/ml in storage buffer (lysis buffer containing 20% glycerol v/v). Molarities of the purified enzymes were determined prior to dilution from UV absorbance measurements and A₂₈₀ extinction coefficients according to established methods (Gill and von Hippel, 1989). These values were consistent with those determined by Bradford assay. Sample aliquots were stored at −80 °C and thawed as needed.

Peptides and reagents

Recombinant human Aβ₁₋₄₀ (rPeptide, Athens, GA, USA), human insulin and insulin B-chain (Sigma-Aldrich, St. Louis, MO, USA) and the quenched fluorogenic aminobenzoic acid (Abz)-SEKKDNYIIKGV-nitroY-OH peptide (AnaSpec, San Jose, CA, USA) were all reconstituted from dry powders. Detailed preparation of Aβ₁₋₄₀ and insulin solutions has been reported elsewhere (Alper and Schmidt, 2009). In brief, Aβ₁₋₄₀ was treated with hexafluoroisopropanol (HFIP) to ensure the initial monomeric state of the peptide (Dahlgren *et al.*, 2002) and dissolved within dimethylsulphoxide (DMSO) to a final concentration of 4 mg/ml. Insulin was resuspended in DMSO to 4 mg/ml and sonicated using a bath sonicator (Branson, Danbury, CT, USA) for approximately 5 min to fully dissolve the peptide. Insulin B-chain was resuspended in DMSO to a final concentration of 4 mg/ml, but did not require sonication for complete solubility. The Abz-SEKKDNYIIKGV-nitroY-OH peptide was resuspended directly within DMSO to a final concentration of 10 mM. After reconstitution, all peptide-containing solutions were stored at −80 °C. Ethylenediaminetetra-acetic acid (EDTA), N-ethylmaleimide (NEM), free cysteine, β-mer-captoethanol (β-ME), dithiothreitol

(DTT), adenosine-5'-triphosphate (ATP) and guanosine triphosphate (GTP) were prepared as 10 mM stocks in deionized water. *N, N, N*'-tetrakis(2-pyridylmethyl)ethylenediamine (TPEN), 1,10-phenanthroline (1,10-P), 4,7-phenanthroline (4,7-P) and phenylmethylsulphonyl fluoride (PMSF) were prepared as 50 mM stocks in DMSO.

Capillary electrophoresis

A β 1–40 and insulin B-chain proteolysis were analysed by capillary electrophoresis (CE) as previously described (Alper and Schmidt, 2009). In brief, proteolytic reactions (40 μ l) were conducted in CE proteolysis buffer (10 mM potassium phosphate, pH 7.6) at 30 °C (Ste23p) or at 37 °C (*Rnl*DE), using 20 nM enzyme and an initial substrate concentration of 0.1 mg/ml (A β 1–40 and insulin B-chain). Enzyme and substrate mixes were independently prepared as 2 $\times$ reaction premixtures by dilution within CE proteolysis buffer that was warmed at 30 °C or 37 °C for 10 min, prior to mixing the premixtures within a prewarmed PCR tube to initiate the proteolytic reaction. Proteolysis was terminated after appropriate intervals by heating the reaction mixture to 80 °C for 3 min. The samples were then stored at –80 °C prior to analysis.

Fluorescence assay

An internally quenched fluorogenic dodecapeptide substrate centred on the M16A proteolytic cleavage site within the yeast P2 a-factor mating pheromone (Abz – SEKKDNYIHKGV–nitroY-OH) was used to monitor the activity of 6 $\times$ His-Ste23p_{M53–E1027} and 6 $\times$ His-IDE_{M42–L1019} under steady-state reaction conditions. This substrate contains a fluorescent aminobenzoic acid moiety (Abz) that is linked through the peptide backbone to a nitrotyrosine (nitroY) fluorescence quencher. Proteolytic cleavage results in enhanced sample fluorescence, which can be quantified using a fluorimeter.

Effects of various environmental conditions upon the enzymatic activities of Ste23p and *Rnl*DE were evaluated by manipulating a component condition of the fluorescence assay reaction mixture while holding all others constant. The standard conditions for assays containing Ste23p (20 nM) were 100 mM potassium phosphate, pH 8.1, and 30 °C; the standard conditions for *Rnl*DE (50 nM) were 100 mM potassium phosphate, pH 9.2, and 37 °C. Potassium phosphate buffers were prepared according to published methods (Gomori, 1956). In general, the use of phosphate buffers was found to permit maximal enzymatic activity relative to alternative buffering systems (Alper, Rowse and Schmidt, unpublished observations). The initial Abz–SEKKDNYIHKGV–nitroY-OH peptide concentration was 17 μ M. Kinetic parameters were determined using the standard conditions described above and initial substrate concentrations in the range 3–172 μ M.

Typical assembly of the reaction mixture involved 1:1 mixing of appropriately diluted substrate and enzyme solutions within an individual well of a prewarmed, black, flat-bottomed fluorescence microtitre plate. The fluorogenic substrate was prepared as a 2 $\times$ reaction pre-mixture by dilution with appropriate buffer in a PCR tube and pre-equilibration for 10 min at the desired temperature. Ste23p and *Rnl*DE were similarly prepared. Where noted, pre-mixtures contained competing substrate (0.16 mg/ml insulin) or added compounds (2 mM), the latter preincubated with the enzyme for 10 min. Proteolysis was

initiated by combining the $2 \times$ premixtures. The total reaction volume of a typical assay was 100 μ l, except in the instance of experiments involving K_m determination, where the total volume was 30 μ l in an effort to permit conservation of substrate. Larger volume samples were combined in a 96-well microtitre plate; smaller volumes were combined in a 384-well plate. Sample fluorescence was analysed at 420 nm over multiple time points over a 60 min time course, using a Bio-Tek[®] Synergy[™] HT fluorimeter equipped with a 320/420 nm excitation/emission filter set (BioTek[®] Instruments, Winooski, VT, USA).

As controls for the fluorescence assay, background and maximum fluorescence signals were determined as reference points for all conditions evaluated. This analysis was performed to identify reaction conditions that modulated the fluorescence of the reporter. The background fluorescence signal was determined in the absence of added enzyme while retaining all other components of the experimental reaction mixture described above. The maximum fluorescence signal was determined in the presence of trypsin (5 μ g/ml), which cleaves the Abz-SEKKDNYIIKGV-nitroY-OH substrate, after the proteolytic reaction was allowed to proceed to completion. In instances where fluorescence was affected (e.g. altered pH, certain added compounds), the absolute difference between the negative and positive controls was used as a normalization factor to calculate the relative fluorescence output of the sample. Rate values observed in the presence of added compounds are reported relative to those for the untreated enzymes.

Rate calculations

Data obtained by fluorescence assay were analysed using Microsoft Excel[™] and GraphPad Prism[™] 4.0 software. Reaction velocity was determined 10–30 min after the initiation of each experiment or up to such time as a maximum of 10% substrate proteolysis was achieved, allowing for an initial lag period upon mixing of the enzyme and substrate premixtures. Reported values represent the average of least three experimental replicates, each derived from 10 experimental data points satisfying the conditions described above. Maximal reaction velocity and initial reaction velocity were taken to be equivalent for purposes of this analysis. Kinetic parameters for Ste23p and RnlDE were determined using non-linear regression curve-fitting software within Prism[™] and a four-parameter logistical equation without constraints.

Because significant intermolecular quenching effects were observed with the fluorescence-based substrate at concentrations $>23 \mu$ M, activities observed at these concentrations were adjusted by correction factors for the purposes of kinetic analysis. These factors were concentration-dependent and derived by comparing best-fit equations for both free Abz and a 1:1 mixture of Abz and nitroY over a range of concentrations. Free Abz data points were best fitted by a second-order polynomial function ($R^2 = 0.9998$), whereas data for the Abz and nitroY mixture were best fitted by a four-parameter logistical equation ($R^2 = 1.000$). Correction factors were determined from ratios of fluorescence (i.e. $\text{RFU}_{\text{Abz}}/\text{RFU}_{\text{Abz}+\text{nitroY}}$) at specific concentrations, which were multiplied against the activities observed at those concentrations. The correction factors were minor (0.98–1.05) between 0–23 μ M and progressively increased (1.09–1.51) over the range 34–172 μ M. Similar mathematical

corrections have been reported for other datasets where intermolecular quenching has been observed (Lazure *et al.*, 1998).

Yeast mating assay

Mating tests were performed essentially as described previously (Alper *et al.*, 2006; Kim *et al.*, 2005). In brief, *MATa* yeast of the indicated genotypes were cultured to saturation in selective (SC-ura) liquid medium, while the *MATa* mating tester (IH1783; *MATa lysI*) was cultured in YEPD. The saturated cultures were then diluted with fresh medium to achieve a cell culture density of 0.95–1.05 OD₆₀₀. The diluted *MATa* cell suspension (98 µl) and an appropriate *MATa* suspension (2 µl) were mixed, and the mixed suspension spotted (5 µl) onto synthetic defined (SD) solid medium. Mating was recorded after incubation at 30 °C for 48–72 h. An equivalent portion of the mixed suspension was also spotted on SC-lys solid medium to serve as a loading control for *MATa* cell input.

Preparation of cell extracts and immunoblotting

Yeast total cellular protein extracts were prepared as previously described (Fujimura-Kamada *et al.*, 1997). In brief, mid-log cells (2 ml 1.0 OD₆₀₀ culture) were harvested by centrifugation, rinsed with deionized water, resuspended in 1 ml cold deionized water and treated with a mixture of NaOH (0.24 N final) and β-mercaptoethanol (0.14 M final) for 15 min while on ice. Proteins were precipitated by addition of TCA (11.5% final), recovered by centrifugation (16 000 × *g* for 15 min), resuspended in 50 µl prewarmed (~100°C) urea sample buffer (250 mM Tris, pH 8.0, 6 M urea, 4% SDS and 0.01% bromophenol blue) and boiled for 3 min. Samples were subjected to SDS–PAGE using a 10% polyacrylamide gel. Immunoblot analysis was performed using anti-HA (Roche, Basel, Switzerland) or anti-Act1p primary antibodies (gift of R. Meager, University of Georgia, Athens, GA, USA), and appropriate HRP-conjugated secondary antibodies (GE Healthcare). Visualization was achieved by chemiluminescent detection methods (BM Chemiluminescence Blotting Substrate, Roche) according to the manufacturer's instructions.

Results

Ste23p is the most highly conserved M16A enzyme within *S. cerevisiae*

Two M16A enzymes are encoded within the *S. cerevisiae* genome: Ste23p and Axl1p. Primary sequence alignments reveal that Ste23p shares a higher degree of amino acid sequence identity with mammalian IDEs than does Axl1p (35% vs. 18% identity to *HsIDE*, respectively; Table 3). Moreover, Ste23p shares roughly equal sequence conservation to mammalian IDEs as is shared by other fungal M16A enzymes, and has higher identity than Axl1p when compared to IDE orthologues from other species. Observations from prior studies further indicate that Ste23p is the most representative IDE orthologue in *S. cerevisiae*, with Axl1p likely having evolved to serve multiple functions that are specific to this organism (see Discussion). Hence, Ste23p was chosen as the model yeast M16A enzyme for the purposes of the current analysis. A primary sequence alignment of Ste23p and *RnIDE* reveals notable features of the two enzymes, including conserved residues of the characteristic M16A motif (Figure 1).

Purification of Ste23p and RnIDE

6×His-Ste23p_{M53-E1027} and 6×His-*RnIDE*_{M42-L1019} were isolated primarily as single protein species of the expected molecular mass (Figure 2). The purified proteins retain N-terminal polyhistidine tags, as evidenced by immunoblot analysis using an anti-6×His antibody (Alper, Rowse and Schmidt, unpublished observations).

Ste23p and *RnIDE* are annotated in public sequence databases as polypeptides having lengths of 1027 and 1019 amino acids, respectively (Cherry *et al.*, 1998; Leinonen *et al.*, 2004). Relative to the annotated Ste23p and *RnIDE* sequences, N-terminally truncated forms of these enzymes were isolated for the purposes of our analysis. Our purification of ‘truncated’ enzymes - beginning, in each case, at the first in-frame methionine encoded downstream from the annotated initiator - over ‘full-length’ polypeptides was motivated by several factors. First, *HsIDE* was recently purified and crystallized as a similarly truncated protein (Shen *et al.*, 2006) and, to our knowledge, there has not been definitive assignment of the initiator methionine for any mammalian IDE (Baumeister *et al.*, 1993). Second, attempts to purify the full annotated sequence of Ste23p as a C-terminal polyhistidine fusion protein led to a purification product having T54 at its N-terminus, as determined by Edmund degradation analysis (Alper, Rowse and Schmidt, unpublished observations); M53 was presumably absent due to the action of methionine aminopeptidase. Third, the ‘truncated’ form of Ste23p most likely represents the physiologically relevant form of the enzyme, as supported by data presented elsewhere within this study (see Figure 6).

Ste23p proteolyses mammalian IDE substrates Aβ1–40 and insulin B-chain

Ste23p has no recognized physiological substrates other than the yeast P2 a-factor; however, the fungal enzyme is capable of proteolysing the established mammalian IDE substrates Aβ1–40 and insulin B-chain (Figure 3). Aβ1–40 and insulin B-chain are common substrates of the M16A subfamily. These peptides have been used to study the proteolytic specificity of M16A enzymes, even in instances where they are unlikely biological targets (Becker and Roth, 1992; Ding *et al.*, 1992; Huet *et al.*, 2008). Analysis of samples from time-course proteolysis experiments shows the accumulation of multiple peaks that presumably correspond with Aβ1–40 and insulin B-chain degradation products, indicating that Ste23p proteolyses each of these substrates at multiple sites (Figures 3B, C). These products are not observed in the presence of histidine-tagged glutathione *S*-transferase (GST-6×His) under otherwise similar reaction conditions (Alper, Rowse and Schmidt, unpublished observations), indicating that proteolysis is not attributable to a contaminating copurification product from the bacterial lysate. Detectable accumulation of Aβ1–40 and insulin B-chain cleavage products is apparent at the 1 and 4 h timepoints, respectively, suggesting that degradation of Aβ1–40 occurs relatively more rapidly. For both substrates, Ste23p-mediated product formation appears slower than that mediated by *RnIDE*, which cleaves Aβ1–40 and insulin B-chain to near completion within 4 h (Figure 3D, E). The identity of Aβ1–40 and insulin B-chain degradation products was not determined during this study. However, *RnIDE* is known to proteolyse both peptides at multiple cleavage sites, which have been previously evaluated (Duckworth *et al.*, 1998; McDermott and Gibson, 1997; Mukherjee *et al.*, 2000).

Comparative activity studies

The enzymatic properties of Ste23p and *RnlDE* were compared using a continuous readout fluorescence assay under conditions of varying pH, salt concentration, temperature and substrate concentration (Figure 4). Both enzymes were most active within alkaline environments (Figure 4A, B), with Ste23p having a maximal activity of 0.49 ± 0.031 $\mu\text{M}/\text{mg}/\text{min}$ at pH 8.1, and *RnlDE* having maximal activity of 0.22 ± 0.004 $\mu\text{M}/\text{mg}/\text{min}$ at pH 9.2, the most alkaline condition evaluated. These values may be compared with the reported values for *RnlDE*-mediated proteolysis of a distinct synthetic fluorogenic Abz–GFLRKGVQ–EDDnp peptide, which exhibits a V_{max} of ~ 0.2 $\mu\text{M}/\text{min}/\text{mg}$, and *RnlDE*-mediated cleavage of β -endorphin, which exhibits a V_{max} of 2.6 $\mu\text{M}/\text{min}/\text{mg}$, albeit under different assay conditions (Song *et al.*, 2001). Both enzymes displayed enhanced enzymatic activity at high salt concentrations, with Ste23p and *RnlDE* activities being stimulated two- and sixfold, respectively, at the highest concentration of potassium chloride evaluated (Figure 4C, D). Both enzymes displayed optimal activity at or about 37 °C (Figure 4E, F). Kinetic parameters for Ste23p and *RnlDE* were determined, revealing that both had roughly equivalent affinity for the Abz–SEKKDNYIIKGV–nitroY-OH peptide under the conditions evaluated (Figure 4G, H). Observed K_m values were 215 ± 69 and 219 ± 22 μM for Ste23p and *RnlDE*, respectively. Negligible activity was observed by fluorescence assay using a GST-6 $\times$ His control (Alper, Rowse and Schmidt, unpublished observations).

Given the otherwise similar enzymatic properties of Ste23p and *RnlDE* identified within our studies, we sought to determine whether the two enzymes exhibited comparable sensitivity to known modulators of M16A peptidase activity, including metal chelators, thiol modifiers and certain inhibitors of cysteine protease activity. This analysis, which was conducted using the fluorescence assay, demonstrated that the sensitivities of Ste23p generally paralleled that observed for *RnlDE* (Figure 5). Addition of EDTA, TPEN and 1,10-phenanthroline, as well as NEM, free cysteine and DTT, resulted in substantial inhibition of both Ste23p and *RnlDE* proteolysis (<40% activity). By comparison, the non-chelating agent 4,7-phenanthroline had limited impact (>60% activity), and addition of β -mercaptoethanol had little or no effect on either enzyme (>80% activity). The serine protease inhibitor PMSF and physiological cofactors ATP and GTP also had limited impact on the activity of both enzymes (>60% activity). Addition of the competing substrate insulin to the proteolytic reaction mixture substantially inhibited both enzymes (<40% activity) but had a more pronounced affect on *RnlDE*.

The predicted initiator methionine of Ste23p is dispensable for activity *in vivo*

Our biochemical analysis revealed that the extreme N-terminus (residues 1–52) of the publicly annotated Ste23p polypeptide sequence is not essential for catalytic function *in vitro*. To better understand the significance of this region *in vivo*, genetic and biochemical methods were used to assess its importance for expression of functional Ste23p (Figure 6). Our standard vector for Ste23p expression contains approximately 1.5 kb genomic sequence 5' of the *STE23* ORF, inclusive of a distinct ORF of opposite orientation that is separated from *STE23* by a 405 base-pair intergenic region (Figure 6A). A modified version of this expression vector that eliminated the entire upstream sequence relative to the 5' margin of the *STE23* ORF (-1467 $\rightarrow$ -1) supported functional Ste23p expression as well as the

unmodified plasmid (Figure 6B, C); the reduced mating observed relative to WT is due to the absence of *AXL1* in the test strain. Loss of additional nucleotide sequence (-1467 → +156), specifically that encoding the annotated initiator codon (M1) and genomic sequence up to but not including the next available methionine codon (M53), abolished Ste23p expression and function, suggesting that the genetic region +1 → +156 of *STE23* is important for gene expression.

We next evaluated the dependence of Ste23p expression on M1 and M53 codons by site-directed mutagenesis (Figure 6D). Mating and protein expression were observed with the M1A mutant but not with the M53A mutant, implying that M53, but not M1, is necessary for the expression of functional Ste23p.

Ste23p is expressed in both haploid and diploid cell types

Whereas expression of the yeast M16A homologue Axl1p is specific to haploid cells (Figure 7B) (Fujita *et al.*, 1994), the expression pattern of Ste23p has not previously been reported. In view of the widespread occurrence of IDE across species and the broad tissue expression of M16A enzymes within multicellular organisms (Baumeister *et al.*, 1993), we hypothesized that Ste23p would be expressed in an unrestricted manner among the three cell types of *S. cerevisiae* — diploid, *MAT^a* and *MAT^α* haploid. Indeed, our analysis indicates that Ste23p is expressed in both diploid and haploid cell types (Figure 7A). Moreover, Ste23p expression levels appear relatively constant within each of the cell types evaluated. Ste23p is produced at levels that are approximately 10-fold higher than Axl1p, as judged by immunoblots of serially diluted samples (Alper *et al.*, 2006). We interpret the unrestricted expression of Ste23p and its high level of production relative to Axl1p as potential indicators of an as-yet undetermined function for Ste23p that is distinct from the enzyme's role in P2 *a*-factor processing. This function must be non-essential, as yeast deficient for *STE23* and/or *AXL1* are viable.

Discussion

The M16A subfamily is emerging as a remarkable class of enzymes, most notably due to the proposed role of human IDE in protecting against Alzheimer's disease and the unusual mechanism by which M16A enzymes capture and cleave their substrates (Selkoe, 2001; Shen *et al.*, 2006). Despite their widespread species distribution, the biological functions of M16A enzymes remain poorly elucidated. Studies of M16A enzymes within well-characterized models may thus serve to distinguish functions of the M16A subfamily that have yet to be identified. In this study, we provide evidence that such studies in yeast should focus on Ste23p rather than Axl1p. This conclusion is based on the higher degree of amino acid sequence conservation that is shared between Ste23p and other M16A enzymes and the remarkably similar biochemical properties of Ste23p and *Rnl*IDE.

To our knowledge, this study presents the first biochemical characterization of a fungal M16A enzyme. We have demonstrated that Ste23p is capable of proteolysing A β 1–40 and insulin B-chain (Figure 3). While neither substrate represents a likely physiological target of the fungal enzyme, these observations are significant in that they provide the first evidence that Ste23p exhibits conserved specificity towards these established substrates of

mammalian M16A enzymes. Reciprocal substrate specificity thus emerges as a general property of fungal, mammalian and prokaryotic M16A enzymes, as we have previously demonstrated that *RnlDE* and *EcPtr* can proteolyse a Ste23p substrate, viz. the P2 precursor of the **a**-factor mating pheromone (Alper *et al.*, 2006; Kim *et al.*, 2005). Our observation that Ste23p can proteolyse both A β 1–40 and insulin B-chain also indicates that substrate recognition by Ste23p does not require the presence of a farnesyl moiety, which is associated with P2 **a**-factor, the only previously recognized substrate of the yeast enzyme. Collectively, these observations led us to develop a fluoro-genic substrate (Abz–SEKKDNYIHKGV–nitroY-OH), based on the amino acid sequence of P2 **a**-factor but lacking the farnesyl moiety of the native mating propheromone. This substrate provides a useful tool for cross-species enzymological studies of the M16A subfamily.

Using a continuous fluorescence assay to measure Ste23p and *RnlDE*-mediated proteolysis of the Abz–SEKKDNYIHKGV–nitroY-OH peptide, we have demonstrated that, in addition to exhibiting reciprocal substrate specificity, Ste23p and *RnlDE* have similar activity profiles in response to alterations in pH, salt concentration and temperature (Figure 4) and various agents that are known modulators of M16A activity (Figure 5). M16A enzymes are recognized as Zn²⁺ metallopeptidases (Becker and Roth, 1992; Ding *et al.*, 1992; Ebrahim *et al.*, 1991; Huet *et al.*, 2008). Consistent with expectations, Ste23p and *RnlDE* were both inhibited by certain metal-chelating agents. Also, like *RnlDE* and other eukaryotic M16A enzymes but unlike bacterial *EcPtr*, Ste23p was inhibited by certain thiol modifiers (Affholter *et al.*, 1990; Becker and Roth, 1992; Ding *et al.*, 1992; Huet *et al.*, 2008; Kuo *et al.*, 1990). Neither Ste23p nor *RnlDE* was sensitive to ATP or GTP addition under the conditions of our analysis. This observation may be interpreted in the context of reports that alternatively claim activation or inhibition of mammalian IDE by these cofactors (Camberos, 2001; Huet *et al.*, 2008; Song *et al.*, 2004). Ste23p also exhibits a broad pH profile, having optimal activity over the pH range 7.6–9.2. Like *RnlDE*, the yeast enzyme was found to be most active under alkaline conditions. We speculate that basic environments may enhance the propensity of M16A substrates to adopt a β -turn structure, which could enhance their interaction with catalytic residues in the N-terminal substrate binding pockets of Ste23p or *RnlDE* or, alternatively, that rate-limiting conformational dynamics within the M16A enzymes themselves are somehow altered under alkaline conditions. The broad pH profile exhibited by Ste23p is not without precedent. Members of the M4 metallopeptidase subfamily (e.g. thermolysin) also exhibit a broad pH profile and are optimally active at alkaline pH (pH 7.0–8.5; Feder and Schuck, 2004). We speculate that the broad range of pH optima exhibited by these enzymes may reflect their having evolved to retain maximal activity under a wide range of environmental conditions for reasons that have yet to be elucidated. Despite the similar biochemical and enzymatic properties of *RnlDE* and Ste23p, whether Ste23p represents a true insulinase remains open to interpretation. While the yeast enzyme pro-teolyse insulin B-chain (Figure 3), we were unable able to document cleavage of intact insulin by Ste23p, despite measurable inhibition of Ste23p-mediated proteolysis upon addition of insulin to the fluorogenic assay mixture (Alper, Rowse and Schmidt, unpublished observations; Figure 5).

Available evidence collectively indicates that Ste23p is the most representative M16A enzyme within *S. cerevisiae*. First, Ste23p shares higher primary sequence homology to mammalian IDEs and other M16A enzymes than does Ax11p (36% vs. 18% identity to HsIDE, respectively; Table 3). Second, like yeast-expressed RnIDE and EcPtr, Ste23p is incapable of supporting certain Ax11p-dependent functions within *S. cerevisiae* (i.e. maintenance of the haploid-specific axial budding pattern and repression of haploid invasive growth (Alper *et al.*, 2006; Cullen and Sprague, 2002; Fujita *et al.*, 1994; Kim *et al.*, 2005). Third, Ste23p has a relatively minor role in **a**-factor processing, despite being overexpressed approximately 10-fold relative to Ax11p in *MAT^a* haploids (Adames *et al.*, 1995; Alper *et al.*, 2006). We now demonstrate that Ste23p is expressed in diploid and *MAT^a* haploid yeast that are incapable of producing **a**-factor (Figure 7). This observation is consistent with the broad tissue expression of RnIDE mRNA transcripts (Baumeister *et al.*, 1993) and may ultimately imply a role for Ste23p in a conserved housekeeping function that has yet to be established.

The assignment of Ste23p as the most representative yeast M16A orthologue supports a continued focus on the biological properties of this enzyme. A number of bioinformatic, genetic and biochemical studies have identified potential interactors of Ste23p that may inform its physiological relevance. However, to our knowledge, none of these interactions has been thoroughly investigated. Any future investigation of Ste23p interactors must take into account two observations. First, we have previously reported that the *STE23* stop codon was misannotated in the original release of the yeast genome sequence (Cherry *et al.*, 1998, Kim, 2005); a similar conclusion was reached through comparative analysis of related fungal genomes (Brachat *et al.*, 2003). The net effect of this misannotation is a predicted translation product with a shortened and altered C-terminus. Second, we now report that the initiator codon of Ste23p is also misannotated (Figure 6). Previously identified genetic interactors of Ste23p must therefore be viewed with caution, as the genetic constructs used to identify these interactors have likely encoded forms of Ste23p with non-physiological N- and C-termini.

In sum, this study provides evidence of extensive enzymatic similarities among Ste23p, RnIDE and other M16A enzymes. These findings support the conclusion that Ste23p represents a suitable model for studies of the M16A subfamily within *S. cerevisiae*. We thus anticipate that continuing studies of Ste23p will serve to provide significant insight into the biological properties of this and other M16A enzymes.

Acknowledgments

We are grateful to Drs Michael Adams, Sidney Kushner, Richard Meagher, Alan Przybyla, Nandu Menon and Zachary Wood, and rPeptide Inc., all of the University of Georgia (Athens, GA, USA), for reagents, access to experimental equipment, contributory efforts and critical discussions that were instrumental to this study. We also thank Dr Surya Manandhar, Marissa Ludley, Jonathan Phillips and other members of Schmidt Lab (UGA), for their contributions to this work.

References

Adames N, Blundell K, Ashby MN, Boone C. Role of yeast insulin-degrading enzyme homologs in propheromone processing and bud site selection. *Science*. 1995; 270:464–467. [PubMed: 7569998]

- Affholter JA, Hsieh CL, Francke U, Roth RA. Insulin-degrading enzyme: stable expression of the human complementary DNA, characterization of its protein product, and chromosomal mapping of the human and mouse genes. *Mol Endocrinol*. 1990; 4:1125–1135. [PubMed: 2293021]
- Alper B, Schmidt W. A capillary electrophoresis method for evaluation of A β proteolysis *in vitro*. *J Neurosci Methods*. 2009; 178:40–45. [PubMed: 19071160]
- Alper BJ, Nienow TE, Schmidt WK. A common genetic system for functional studies of pitrilysin and related M16A proteases. *Biochem J*. 2006; 398:145–152. [PubMed: 16722821]
- Baumeister H, Muller D, Rehbein M, Richter D. The rat insulin-degrading enzyme. Molecular cloning and characterization of tissue-specific transcripts. *FEBS Lett*. 1993; 317:250–254. [PubMed: 8425612]
- Becker AB, Roth RA. An unusual active site identified in a family of zinc metalloendopeptidases. *Proc Natl Acad Sci USA*. 1992; 89:3835–3839. [PubMed: 1570301]
- Becker AB, Roth RA. Identification of glutamate-169 as the third zinc-binding residue in proteinase III, a member of the family of insulin-degrading enzymes. *Biochem J*. 1993; 292(pt 1):137–142. [PubMed: 8099278]
- Becker AB, Roth RA. Insulysin and pitrilysin: insulin-degrading enzymes of mammals and bacteria. *Methods Enzymol*. 1995; 248:693–703. [PubMed: 7674956]
- Bennett RG, Duckworth WC, Hamel FG. Degradation of amylin by insulin-degrading enzyme. *J Biol Chem*. 2000; 275:36621–36625. [PubMed: 10973971]
- Bieri T, Blasiar D, Ozersky P, et al. WormBase: new content and better access. *Nucleic Acids Res*. 2007; 35:D506–510. [PubMed: 17099234]
- Bondy CA, Zhou J, Chin E, et al. Cellular distribution of insulin-degrading enzyme gene expression. Comparison with insulin and insulin-like growth factor receptors. *J Clin Invest*. 1994; 93:966–973. [PubMed: 8132782]
- Brachat S, Dietrich FS, Voegeli S, et al. Reinvestigation of the *Saccharomyces cerevisiae* genome annotation by comparison to the genome of a related fungus: *Ashbya gossypii*. *Genome Biol*. 2003; 4:R45. [PubMed: 12844361]
- Broad Institute of Harvard and MIT. *Ustilago maydis* Sequencing Project. 2008.
- Camberos MC, Perez AA, Udrișar DP, et al. ATP inhibits insulin-degrading enzyme activity. *Exp Biol Med*. 2001; 226:334–341.
- Cherry JM, Adler C, Ball C, et al. SGD: *Saccharomyces* genome database. *Nucleic Acids Res*. 1998; 26:73–79. [PubMed: 9399804]
- Cullen PJ, Sprague GF Jr. The roles of bud-site-selection proteins during haploid invasive growth in yeast. *Mol Biol Cell*. 2002; 13:2990–3004. [PubMed: 12221111]
- Dahlgren KN, Manelli AM, Stine WB, et al. Oligomeric and fibrillar species of amyloid-peptides differentially affect neuronal viability. *J Biol Chem*. 2002; 277:32046–32053. [PubMed: 12058030]
- Ding L, Becker AB, Suzuki A, Roth RA. Comparison of the enzymatic and biochemical properties of human insulin-degrading enzyme and *Escherichia coli* protease III. *J Biol Chem*. 1992; 267:2414–2420. [PubMed: 1733942]
- Duckworth WC, Bennett RG, Hamel FG. Insulin degradation: progress and potential. *Endocr Rev*. 1998; 19:608–624. [PubMed: 9793760]
- Ebrahim A, Hamel FG, Bennett RG, Duckworth WC. Identification of the metal associated with the insulin degrading enzyme. *Biochem Biophys Res Commun*. 1991; 181:1398–1406. [PubMed: 1764090]
- Elble R. A simple and efficient procedure for transformation of yeasts. *BioTechniques*. 1992; 13:18–20. [PubMed: 1503765]
- Elia L, Marsh L. Role of the ABC transporter Ste6 in cell fusion during yeast conjugation. *J Cell Biol*. 1996; 135:741–751. [PubMed: 8909547]
- Ertekin-Taner N, Graff-Radford N, Younkin LH, et al. Linkage of plasma A42 to a quantitative locus on chromosome 10 in late-onset Alzheimer's disease pedigrees. *Science*. 2000; 290:2303–2304. [PubMed: 11125143]

- Farris W, Mansourian S, Chang Y, et al. Insulin-degrading enzyme regulates the levels of insulin, amyloid β -protein, and the β -amyloid precursor protein intracellular domain *in vivo*. *Proc Natl Acad Sci USA*. 2003; 100:4162–4167. [PubMed: 12634421]
- Feder J, Schuck M. Comparative kinetic studies on the neutral protease and thermolysin-catalyzed hydrolysis of simple dipeptide substrates. *Biochemistry*. 1970; 9:2784–2791. [PubMed: 4989948]
- Fujimura-Kamada K, Nouvet FJ, Michaelis S. A novel membrane-associated metalloprotease, Ste24p, is required for the first step of NH₂-terminal processing of the yeast a-factor precursor. *J Cell Biol*. 1997; 136:271–285. [PubMed: 9015299]
- Fujita A, Oka C, Arikawa Y, et al. A yeast gene necessary for bud-site selection encodes a protein similar to insulin-degrading enzymes. *Nature*. 1994; 372:567–570. [PubMed: 7990931]
- Gill S, von Hippel P. Calculation of protein coefficients from amino acid sequence data. *Anal Biochem*. 1989; 182:319–326. [PubMed: 2610349]
- Gomori G. Preparation of buffers for use in enzyme studies. *Methods Enzymol*. 1956; 1:138–146.
- Groves CJ, Wiltshire S, Smedley D, et al. Association and haplotype analysis of the insulin-degrading enzyme *IDE* gene, a strong positional and biological candidate for type 2 diabetes susceptibility. *Diabetes*. 2003; 52:1300–1305. [PubMed: 12716770]
- Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol*. 1983; 166:557–580. [PubMed: 6345791]
- Hardy J, Selkoe DJ. The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics. *Science*. 2002; 297:353–356. [PubMed: 12130773]
- Huet Y, Strassner J, Schaller A. Cloning, expression and characterization of insulin-degrading enzyme from tomato *Solanum lycopersicum*. *Biol Chem*. 2008; 389:91–98. [PubMed: 18095874]
- Im H, Manolopoulou M, Malito E, et al. Structure of substrate-free human insulin-degrading enzyme IDE and biophysical analysis of ATP-induced conformational switch of IDE. *J Biol Chem*. 2007; 282:25453–25463. [PubMed: 17613531]
- Kim M, Hersh LB, Leissring MA, et al. Decreased catalytic activity of the insulin degrading enzyme in chromosome 10-linked Alzheimer's disease families. *J Biol Chem*. 2007; 282:7825–7832. [PubMed: 17244626]
- Kim S, Lapham A, Freedman C, et al. Yeast as a tractable genetic system for functional studies of the insulin-degrading enzyme. *J Biol Chem*. 2005; 280:27481–27490. [PubMed: 15944156]
- Kirschner RJ, Goldberg AL. A high molecular weight metalloendoprotease from the cytosol of mammalian cells. *J Biol Chem*. 1983; 258:967–976. [PubMed: 6401723]
- Kuo WL, Gehm BD, Rosner MR. Cloning and expression of the cDNA for a *Drosophila* insulin-degrading enzyme. *Mol Endocrinol*. 1990; 4:1580–1591. [PubMed: 2126597]
- Kurochkin IV. Amyloidogenic determinant as a substrate recognition motif of insulin-degrading enzyme. *FEBS Lett*. 1998; 427:153–156. [PubMed: 9607302]
- Larkin MA, Blackshields G, Brown NP, et al. Clustal W and Clustal X version 2.0. *Bioinformatics*. 2007; 23:2947–2948. [PubMed: 17846036]
- Lazure C, Gauthier D, Jean F, et al. *In vitro* cleavage of internally quenched fluorogenic human parathyroid hormone and parathyroid-related peptide substrates by furin. Generation of a potent inhibitor. *J Biol Chem*. 1998; 273:8572–8580.
- Leinonen R, Diez FG, Binns D, et al. UniProt archive. *Bioinformatics*. 2004; 20:3236–3237. [PubMed: 15044231]
- Leissring MA, Farris W, Chang AY, et al. Enhanced proteolysis of β -amyloid in APP transgenic mice prevents plaque formation, secondary pathology, and premature death. *Neuron*. 2003; 40:1087–1093. [PubMed: 14687544]
- Li P, Kuo WL, Yousef M, et al. The C-terminal domain of human insulin degrading enzyme is required for dimerization and substrate recognition. *Biochem Biophys Res Commun*. 2006; 43:1032–1037.
- Maskos K, Josic D, Fernandez-Catalan C. Crystal structure of pitrilysin, the prototype of insulin-degrading enzymes. *RCSB PDB*:1Q2L. 2005; doi: 10.2210/pdb1Q2L/pdb
- McDermott JR, Gibson AM. Degradation of Alzheimer's β -amyloid protein by human and rat brain peptidases: involvement of insulin-degrading enzyme. *Neurochem Res*. 1997; 22:49–56. [PubMed: 9021762]

- Michaelis S, Herskowitz I. The α -factor pheromone of *Saccharomyces cerevisiae* is essential for mating. *Mol Cell Biol*. 1988; 8:1309–1318. [PubMed: 3285180]
- Miller BC, Eckman EA, Sambamurti K, et al. Amyloid- β peptide levels in brain are inversely correlated with insulin activity levels *in vivo*. *Proc Natl Acad Sci USA*. 2003; 100:6221–6226. [PubMed: 12732730]
- Mukherjee A, Song E, Kihiko-Ehmann M, et al. Insulysin hydrolyzes amyloid- β peptides to products that are neither neurotoxic nor deposit on amyloid plaques. *J Neurosci*. 2000; 20:8745–8749. [PubMed: 11102481]
- Myers A, Holmans P, Marshall H, et al. Susceptibility locus for Alzheimer's disease on chromosome 10. *Science*. 2000; 290:2304–2305. [PubMed: 11125144]
- Neant-Fery M, Garcia-Ordóñez RD, Logan TP, et al. Molecular basis for the thiol sensitivity of insulin-degrading enzyme. *Proc Natl Acad Sci USA*. 2008; 105:9582–9587. [PubMed: 18621727]
- Palecek SP, Parikh AS, Kron SJ. Genetic analysis reveals that FLO11 upregulation and cell polarization independently regulate invasive growth in *Saccharomyces cerevisiae*. *Genetics*. 2000; 156:1005–1023. [PubMed: 11063681]
- Perlman RK, Gehm BD, Kuo WL, Rosner MR. Functional analysis of conserved residues in the active site of insulin-degrading enzyme. *J Biol Chem*. 1993; 268:21538–21544. [PubMed: 8104941]
- Qiu WQ, Walsh DM, Ye Z, et al. Insulin-degrading enzyme regulates extracellular levels of amyloid β -protein by degradation. *J Biol Chem*. 1998; 273:32730–32738. [PubMed: 9830016]
- Rawlings ND, Morton FR, Kok CY, et al. MEROPS: the peptidase database. *Nucleic Acids Res*. 2008; 36:D320–325. [PubMed: 17991683]
- Selkoe DJ. Clearing the brain's amyloid cobwebs. *Neuron*. 2001; 32:177–180. [PubMed: 11683988]
- Seta KA, Roth RA. Overexpression of insulin degrading enzyme: cellular localization and effects on insulin signaling. *Biochem Biophys Res Commun*. 1997; 231:167–171. [PubMed: 9070242]
- Shen Y, Joachimiak A, Rosner MR, Tang WJ. Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism. *Nature*. 2006; 443:870–874. [PubMed: 17051221]
- Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics*. 1989; 122:19–27. [PubMed: 2659436]
- Song E, Daily A, Fried M, et al. Mutation of active site residues of the insulin-degrading enzyme alters allosteric interactions. *J Biol Chem*. 2005; 280:17701–17706. [PubMed: 15749695]
- Song ES, Juliano MA, Juliano L, et al. ATP effects on insulin-degrading enzyme are mediated primarily through its triphosphate moiety. *J Biol Chem*. 2004; 279:54216–54220. [PubMed: 15494400]
- Song ES, Mukherjee A, Juliano MA, et al. Analysis of the subsite specificity of rat insulysin using fluorogenic peptide substrates. *J Biol Chem*. 2001; 276:1152–1155. [PubMed: 11042190]
- Stein L, Sternberg P, Durbin R, et al. WormBase: network access to the genome and biology of *Caenorhabditis elegans*. *Nucleic Acids Res*. 2001; 29:82–86. [PubMed: 11125056]
- Wilbur WJ, Lipman DJ. Rapid similarity searches of nucleic acid and protein data banks. *Proc Natl Acad Sci USA*. 1983; 80:726–730. [PubMed: 6572363]
- Wood V, Gwilliam R, Rajandream MA, et al. The genome sequence of *Schizosaccharomyces pombe*. *Nature*. 2002; 415:871–880. [PubMed: 11859360]

Figure 2.

Purification recombinant Ste23p and *RnIDE*. Polyhistidine tagged Ste23p and *RnIDE* were expressed in *E. coli* from pET expressions vectors (pWS769 and pWS804, respectively) and isolated to near-homogeneity. Two-fold serial dilutions of purified 6×His-Ste23p_{M53-E1027} and 6×His-*RnIDE*_{M42-L1019} were analysed by SDS-PAGE and stained using CoomassieTM brilliant blue R-250. The leftmost lane of each dilution series contains 7.5 µg total protein (lanes 1 and 5). The predicted molecular mass of 6×His-Ste23p_{M53-E1027} is 113 kDa and the predicted mass of 6×His-*RnIDE*_{M42-L1019} is 114 kDa

Figure 3.

Ste23p proteolyses Aβ1–40 and insulin B-chain. The ability of Ste23p and *RnIDE* to proteolyse established IDE substrates Aβ1–40 and insulin B-chain was evaluated by capillary electrophoresis (CE). Shown are chromatogram traces depicting UV absorbance at 200 nm. (A) Analysis of the individual components of the proteolytic reaction mixture. Solutions contain 24 μM Aβ1–40, 30 μM insulin B-chain, 20 nM Ste23p or 20 nM *RnIDE* in proteolysis buffer, reflective of their initial concentrations within the proteolytic reaction mixture. (B, C) Profile of Aβ1–40 (B) and insulin B-chain (C) products produced by Ste23p at indicated timepoints. Peaks contributed by substrates are denoted with arrowheads, and

peaks contributed by the enzymatic experimental component are denoted with asterisks. (D, E) Profile of A β 1–40 (D) and insulin B-chain (E) produced by *RnIDE* after 4 h of incubation

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 4.

Comparative enzymatic properties of Ste23p and RnIDE. Proteolysis of the Abz-SEKKDNYIIKGV-nitroY-OH peptide by Ste23p (A, C, E) and RnIDE (B, D, F) was evaluated over the indicated range of pH (A, B), salt concentration (C, D) and temperature (E, F), using a continuous fluorescence assay. Proteolytic activity rates were determined from initial rates and are presented as percentages of the maximal enzyme activity observed within an experiment for each variable evaluated. Reported values are the average of three experimental replicates. Error bars depict standard deviation from the mean. Velocity vs. substrate concentration curves for Ste23p (G) and RnIDE (H) were determined over a

substrate concentration range of 3–172 μM . K_m values were derived using the non-linear regression function of GraphPad Prism™ 4.0

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 5.

Sensitivity of Ste23p and *RnIDE* to various agents. Activities of Ste23p (black bars) and *RnIDE* (grey bars) were determined as described in Figure 4 in the presence of the indicated agents relative to untreated and DMSO (dimethyl sulphoxide)-treated controls. Reported values are the average of three experimental replicates, and error bars indicate standard deviation from the mean. EDTA (ethylenediaminetetraacetic acid), TPEN (*N, N, N'*-tetrakis-[2-pyridylmethyl]ethylenediamine), 1,10-P (1,10-phenanthroline), 4,7-P (4,7-phenanthroline), NEM (*N*-ethylmaleimide), β-ME (β-mercaptoethanol), DTT (dithiothreitol), PMSF (phenylmethylsulphonylfluoride), ATP (adenosine-5'-triphosphate), and GTP (guanosine-5'-triphosphate) were added to a concentration of 1 mM within the proteolytic reaction mixture. Insulin was added to an initial concentration of 0.8 mg/ml

Figure 6.

Characterization of the translation start site of Ste23p. (A) Schematic of the genomic DNA sequence that is 5' to the predicted start codon of the *STE23* open reading frame within pWS482. Schematic is not drawn to scale. (B) The predicted 5' UTR of *STE23* is not required for expression of functional Ste23p. A set of plasmids bearing deletions of the 5' UTR contained within pWS482 (pWS759-763) and a plasmid lacking part of the *STE23* ORF itself (pWS765) were evaluated for their ability to promote yeast mating (top panel) and Ste23p expression (middle panel). Yeast mating was evaluated by mixing *MATa* cells (IH1793) and M16A-deficient *MATa* cells (Y272) carrying the indicated plasmid, followed by selection on diploid-selective media. Ste23p expression was evaluated by immunoblotting. Equivalent percentage amounts (2%) of total cellular protein extracts were separated by SDS-PAGE and transferred onto blots that were probed with anti-HA and anti-Act1p antibodies as a loading control (middle and bottom panels, respectively). (C) Expression of Ste23p partially rescues the mating defect of an M16A-deficient yeast strain (Y272). Yeast mating assays and analysis of Ste23p expression were performed as described in (B); the panel order is preserved. WT (IH1783) was transformed with an empty vector (pRS316), and Y272 was transformed with either pRS316 or a plasmid encoding Ste23p-2HA (pWS482). (D) The annotated start codon of *STE23* is not required for expression of functional Ste23p. Yeast mating assays and analysis of Ste23p expression were performed as described in (B), using plasmid-transformed Y272 cells, except that 4% of each extract was evaluated; the panel order is preserved. Plasmids used were pWS908-909

Figure 7.

Ste23p is expressed in both haploid and diploid yeast. Plasmid-based expression of Ste23p (A) and Axl1p (B) was examined in *MATa* (IH1783), *MATα* (IH1784) and diploid (IH1788) cell types, using pWS482 and pWS371, respectively. The steady-state levels of the indicated HA-tagged protein (top panel) and yeast actin (bottom panel) were detected by immunoblotting as described in Figure 6, except that 60% of the total cellular extract preparation was evaluated in the instance of Axl1p samples, due to its low abundance

Table 1

Yeast and bacterial strains used in this study

Strain	Genotype ^a	Reference
IH1783	<i>MATa trp1 leu2 ura3 his4 can1</i>	(Michaelis and Herskowitz, 1988)
IH1784	<i>MATa trp1 leu2 ura3 his4 can1</i>	(Adames <i>et al.</i> , 1995)
IH1788	<i>MATa/a trp1 leu2 ura3 his4 can1</i>	(Michaelis and Herskowitz, 1988)
IH1793	<i>MATa lys1</i>	(Michaelis and Herskowitz, 1988)
Y272	<i>MATa trp1 leu2 ura3 his4 can1 ax11::LEU2 ste23::LEU2</i>	(Adames <i>et al.</i> , 1995)
DH5 α	<i>F-f80lacZD(lacZYA-argF) U169deoR recA1 endA1 hsdR17(rk-mk+) phoA supE44 thi-1gyr A96 relA1 ton A</i>	Invitrogen
BL21(DE3)	<i>F⁻ ompT hsdS_B(r_B⁻ m_B⁻) gal dcm (DE3)</i>	Novagen

^a IH1783, IH1784, IH1788 and Y272 are isogenic.

Table 2

Plasmids used in this study

Plasmid	Genotype	Reference
p80	<i>CEN URA3 STE23</i>	(Adames <i>et al.</i> , 1995)
pET-30b ⁺	<i>pBR322 f1 KAN lacI pT7</i>	Novagen
pSM1569	<i>AMP.SRa-RnIDE</i>	(Bondy <i>et al.</i> , 1994; Seta and Roth, 1997)
pRS316	<i>CEN URA3</i>	(Sikorski and Hieter, 1989)
pWS371	<i>CEN URA3 AXLI-2HAc</i>	(Kim <i>et al.</i> , 2005)
pWS375	<i>CEN URA3 STE23</i>	(Kim <i>et al.</i> , 2005)
pWS482	<i>CEN URA3 STE23-2HAc</i>	(Kim <i>et al.</i> , 2005)
pWS759	<i>CEN URA3 STE23-2HAc (-1467 → -1)</i>	This study
pWS760	<i>CEN URA3 STE23-2HAc (-1467 → -100)</i>	This study
pWS761	<i>CEN URA3 STE23-2HAc (-1467 → -200)</i>	This study
pWS762	<i>CEN URA3 STE23-2HAc (-1467 → -300)</i>	This study
pWS763	<i>CEN URA3 STE23-2HAc (-1467 → -400)</i>	This study
pWS765	<i>CEN URA3 STE23-2HAc (-1467 → +156)</i>	This study
pWS769	<i>pBR322 f1 KAN lacI pT7 6×HIS-STE23_{M53-E1027}</i>	This study
pWS804	<i>pBR322 f1 KAN lacI pT7 6×HIS-RnIDE_{MA2-L1019}</i>	(Alper and Schmidt, 2009)
pWS908	<i>CEN URA3 STE23_{M1A}-2HAc</i>	This study
pWS909	<i>CEN URA3 STE23_{M53A}-2HAc</i>	This study

Table 3

Conservation of sequence identity among M16A enzymes^{a,b}

	<i>Hs</i> IDE	<i>Rn</i> IDE	<i>Dm</i> IDE	<i>Ce</i> IDE	<i>Sl</i> IDE	<i>Um</i> Ste23p	<i>Sp</i> Ste23p	<i>Ste</i> 23p	<i>Ec</i> Ptr	<i>Axl</i> lp
<i>Hs</i> IDE	100	94	45	40	38	37	36	35	25	18
<i>Rn</i> IDE		100	45	39	38	38	36	35	24	18
<i>Dm</i> IDE			100	39	35	37	32	34	24	18
<i>Ce</i> IDE				100	31	32	31	32	22	16
<i>Sl</i> IDE					100	36	35	32	24	19
<i>Um</i> Ste23p						100	39	36	26	14
<i>Sp</i> Ste23p							100	36	25	18
<i>Ste</i> 23p								100	24	16
<i>Ec</i> Ptr									100	12
<i>Axl</i> lp										100

^aPercentage sequence identity calculated using multiple global sequence alignment function of Clustal W 2.0.10.

^b*Hs*, Homo sapiens; *Rn*, Rattus norvegicus; *Dm*, Drosophila melanogaster; *Ce*, Caenorhabditis elegans; *Sl*, Solanum lycopersicum; *Um*, Ustilago maydis; *Sp*, Schizosaccharomyces pombe; *Ec*, Escherichia coli.