[11] Biochemical and Genetic Methods for Analyzing Specificity and Activity of a Precursor-Processing Enzyme: Yeast Kex2 Protease, Kexin

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Introduction

The Ca^{2+}-dependent, transmembrane Kex2 protease of the yeast *Saccharomyces cerevisiae* (kexin, EC 3.4.21.61) was discovered through analysis of strains bearing mutations in the *KEX2* gene that blocked post-translational processing of the precursors of two secreted peptides, the α-mating pheromone and M_1 killer toxin.[1-3] Kex2 protease has emerged as the prototype of a family of eukaryotic enzymes that cleave polypeptide precursors, including prohormones, neuropeptide precursors, and the precursors of a variety of secreted and integral membrane proteins, in late compartments of the secretory pathway.[4] Most such cleavages occur at the carboxyl side of pairs of basic residues, especially -Lys-Arg- and -Arg-Arg-, although cleavages also occur at monobasic and polybasic sites. Additional members of the Kex2 family are discussed in [12] and [13] in this volume.[5,6]

Kex2 protease and its metazoan homologs form a distinct branch of the subtilisin family of serine peptidases, previously characterized members of which were degradative enzymes with relatively little substrate discrimination.[7-9] As a prototype of a family of enzymes involved in biosynthetic proteolysis, Kex2 is being investigated to elucidate the kinetic and structural properties that provide the remarkable specificity that these enzymes display in processing polypeptide precursors.

Several factors, divisible into two broad categories, can contribute to the specificity of a processing enzyme within the cell. First, intracellular

[1] M. J. Leibowitz and R. B. Wickner, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 73 (1976).
[2] D. Julius, L. Blair, A. Brake, G. Sprague, and J. Thorner, *Cell (Cambridge, Mass.)* **32**, 839 (1983).
[3] R. S. Fuller, R. E. Sterne, and J. Thorner, *Annu. Rev. Physiol.* **50**, 345 (1988).
[4] D. F. Steiner, S. P. Smeekens, S. Ohagi, and S. J. Chan, *J. Biol. Chem.* **267**, 23,435 (1992).
[5] K. Nakayama, this volume [12].
[6] M. Chretien and N. G. Seidah, this volume [13].
[7] R. S. Fuller, A. J. Brake, and J. Thorner, *Science* **246**, 482 (1989).
[8] R. J. Siezen, W. M. deVos, J. A. M. Leunissen, and B. W. Dijkstra, *Protein Eng.* **4**, 719 (1991).
[9] P. Gluschankof and R. S. Fuller, *EMBO J.* **13**, 2280 (1994).

targeting and cocompartmentalization of enzymes and substrates can limit the range of molecules exposed to an enzyme. Conditions within the processing compartment may further modulate the activity or specificity of the enzyme and the conformation or accessibility of potential substrates. Although these "cellular" factors will not be the subject of this chapter, it is worthy of note that Kex2 protease is localized quite selectively to a late compartment of the Golgi complex in yeast, most likely equivalent to the mammalian trans-Golgi network.[10,11] Retention of Kex2 protease in this compartment requires both its single transmembrane domain and a "retention signal" in the C-terminal cytosolic tail sequence of the enzyme that resembles the tyrosine-internalization signals found in mammalian cell surface receptors.[7,12] Mutation of the retention signal lowers the steady-state level of Kex2 protease in the pro-α-factor processing compartment, resulting in decreased processing efficiency.[12] Clearly for Kex2, and undoubtedly for other processing proteases as well, correct localization within the secretory pathway is critical for biochemical function within the cell.

The second and more obvious set of factors affecting specificity governs the direct physical interaction between the enzyme and substrate. These factors determine the degree of fidelity in substrate discrimination that the enzyme can achieve, ensuring efficient cleavage of correct bonds in intended substrates and not of incorrect bonds in substrates or nonsubstrates. At a first level, the enzyme recognizes substrate residues near the cleaved bond. Such "primary" specificity is ideally probed using small, conformationally unconstrained peptide substrates and inhibitors. However, the physiological substrates of Kex2 and other precursor processing enzymes are polypeptides that presumptively possess both secondary and tertiary structure. The degree to which higher order structural features influence processing specificity is still an open question. One straightforward way to assess the influence of higher order structure is to compare the kinetics of cleavage of a site in a native precursor with the cleavage of a conformationally unconstrained peptide having the corresponding sequence.

The information specifying localization of Kex2 is found in C-terminal sequences that are exclusive of the catalytic domain on which analysis of enzymatic specificity focuses.[7,9,12,13] Thus a genetically engineered, C-terminally truncated form of Kex2 protease has provided a convenient

[10] K. Redding, C. Holcomb, and R. S. Fuller, *J. Cell Biol.* **113**, 527 (1991).
[11] C. A. Wilcox and R. S. Fuller, *J. Cell Biol.* **115**, 297 (1991).
[12] C. A. Wilcox, K. Redding, R. Wright, and R. S. Fuller, *Mol. Biol. Cell* **3**, 1353 (1992).
[13] R. S. Fuller, A. J. Brake, and J. Thorner, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 1434 (1989).

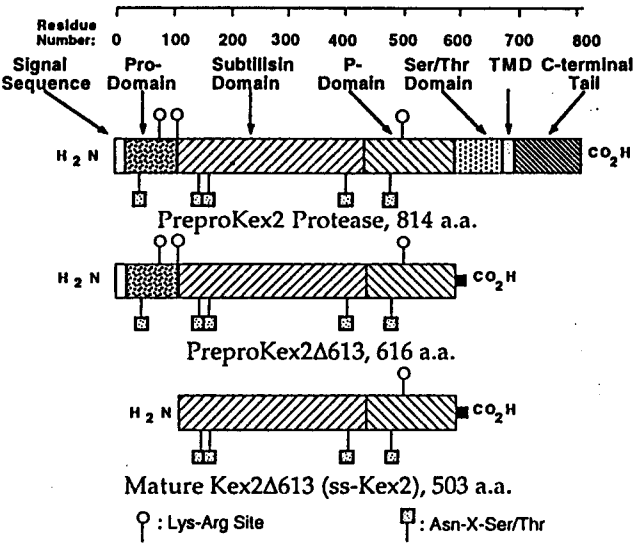

FIG. 1. Schematic structures of prepro-Kex2 protease, prepro-Kex2Δ613, and mature ss-Kex2. Features of the sequence of prepro-Kex2 protease, indicated by arrows, are described in the text. TMD, Transmembrane domain. Prepro-Kex2Δ613 ends with Kex2 residue Glu-613 plus three linker-encoded residues (Arg-Asn-Arg). See P. Gluschankof and R. S. Fuller, *EMBO J.* **13**, 2280 (1994).

model for studies of enzymatic activity and specificity (Fig. 1).[14] Methods of purification, active site titration, and characterization of this secreted, soluble form of Kex2 protease (ss-Kex2) are described in the first three sections of this chapter. We have taken several approaches to studying the enzymatic specificity of Kex2 protease. The uses of peptidyl methylcoumarylamide (MCA) substrates[14] and peptidyl chloromethanes[15] as probes of Kex2 specificity have been described. Such peptides permit analysis of the interactions with substrate residues N-terminal to the cleaved bond; that is, in the P1, P2, etc., positions. Internally quenched fluorogenic peptide substrates permit sensitive and quantitative analysis of the effects of substitutions of residues on both sides of the cleaved bond.[16] Use of such substrates based on cleavage sites in pro-α-factor will be described elsewhere.[17] Certain general conclusions can be drawn from these studies. First, wild-type Kex2 protease exhibits a high degree of

[14] C. Brenner and R. S. Fuller, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 922 (1992).
[15] H. Angliker, P. Wikstrom, E. Shaw, C. Brenner, and R. S. Fuller, *Biochem. J.* **293**, 75 (1993).
[16] E. D. Matayoshi, G. T. Wang, G. A. Krafft, and J. Erickson, *Science* **247**, 954 (1990).
[17] C. Brenner, G. Wang, G. Krafft, and R. S. Fuller, unpublished data (1993).

primary specificity, displaying its highest k_{cat}/K_M of $\sim 10^7\ M^{-1}\ sec^{-1}$ for the substrate acetyl-Pro-Met-Tyr-Lys-Arg-MCA, which is based on three of the four Kex2 cleavage sites in pro-α-factor. Kex2 shows a strict requirement for an Arg residue at P1. With Arg at P1, nearly all substrates exhibit a k_{cat} in the range of 20 to 40 sec^{-1}, whereas Lys at P1 reduces k_{cat} >100-fold and k_{cat}/K_M up to 3000-fold. Substitutions at P2 principally affect K_M, with Lys being preferred to Arg by ~4-fold.

As alluded to previously, analysis of cleavage of small peptides may tell only part of the specificity story, because higher order structure in an authentic precursor may affect its interaction with a processing enzyme. The analysis of cleavage of pro-α-factor by Kex2 protease, both in a reconstituted system and *in vivo*, will be discussed in the fourth and fifth sections of this chapter. The yeast genetic system affords additional tools for the study of Kex2 activity and specificity. Because Kex2 protease catalyzes a required step in synthesis of α-factor, the mating pheromone secreted by haploid cells of the α mating type, *kex2* mutants are sterile.[1,2] This phenotype is easily scored either qualitatively or quantitatively on petri plates by the simple appearance or frequency of appearance of prototrophic a/α diploids from the mating of auxotrophic **a** and α haploids. These bioassays can be used to monitor the effects of mutations in pro-α-factor on Kex2 cleavage, or alternatively, to screen for mutations in the enzyme that affect its activity or specificity. An example of this second approach is provided in the final section on "one-step site-directed mutagenesis" of the Kex2 oxyanion hole.[18]

Methods

Engineering and Purification of Secreted, Soluble Kex2 Protease

Kex2 protease contains a domain of ~155 amino acids, carboxyl to the domain with 30% identity to subtilisin, termed the P domain, which is conserved in metazoan Kex2 homologs and is required for biosynthesis of active Kex2 protease.[9,19] Truncation of the Ser/Thr-rich domain, transmembrane domain, and cytosolic tail carboxyl to the P domain (see Fig. 1) results in secretion of the ss-Kex2 enzyme into culture medium.[9,14] Both wild-type and active, C-terminally truncated forms of prepro-Kex2 undergo intramolecular, autoproteolytic removal of the N-terminal prodo-

[18] C. Brenner, A. Bevan, and R. S. Fuller, *Curr. Biol.* 3, 498 (1993).

[19] R. S. Fuller, C. Brenner, P. Gluschankof, and C. A. Wilcox, *in* "Methods in Protein Sequence Analysis" (H. Jörnvall, J.-O. Hoog, and A.-M. Gustavsson, eds.), p. 205. Birkhaeuser, Basel, 1991.

main by cleavage after Lys[108]-Arg[109] shortly after translocation into the endoplasmic reticulum.[9,11] In the Golgi, two N-terminal dipeptides (Leu[110]-Pro[111] and Val[112]-Pro[113]) are removed by the *STE13*-encoded type IV dipeptidyl-peptidase.[14]

For purposes of purification, the truncated *KEX2* gene, *KEX2Δ613*, is expressed by a powerful constitutive promoter of the *TDH3* gene (glyceraldehyde-3-phosphate dehydrogenase), on the high copy number yeast episomal plasmid pG5 containing the *URA3* gene as a selectable marker.[14] We have found that buffering growth medium to pH 7.2 and inclusion of 0.5% casamino acids (Difco, Detroit, MI) leads to consistently high yields. Growth of ss-Kex2-secreting cells in unbuffered medium leads to extremely low yields of enzyme due to the instability of the enzyme in medium acidified by yeast fermentation. Curiously, growth of ss-Kex2-producing cells to stationary phase in 10-ml quantities in 18 × 150-mm culture tubes, but not in bulk in shake flasks, leads to very high levels of accumulation of active enzyme in culture medium, up to ~30 mg/liter when using yeast strain ABY01. Preparations as large as several liters have been prepared by growing hundreds of 10-ml cultures. The following methods are adapted from Brenner and Fuller.[14]

Preparaton of ss-Kex2

Day 1. The lithium acetate transformation method[20] is used to introduce ss-Kex2-overproducing plasmid pG5-*KEX2Δ613*[14] into strain ABY01 (*MAT***a** *ura3 trp1 ade2 his3*). Transformants are selected by plating at 30° on synthetic complete medium lacking uracil (SDC-Ura).[21]

Day 2. A pump is used to deliver 4 liters of 1040 medium (below) in 10-ml volumes to 18-mm × 150-mm culture tubes, which are then capped and autoclaved.

Day 3. ~10 Ura^+ transformants are inoculated into 2-ml overnight cultures of SDC-Ura medium and grown overnight to saturation at 30° in a Rollordrum (New Brunswick Scientific, Edison, NJ).

Day 4. 1 ml of each saturated SDC-Ura culture is used to inoculate a 10-ml volume of 1040 medium for overnight growth at 30°.

Day 5. 2 μl of a 1 : 10 dilution of culture medium of the 1040 overnights are assayed for Kex2 activity (below). Cultures exhibiting ≥300–400 U/μl of medium are used to inoculate the remaining 10-ml volumes of 1040 medium at a 1 : 20 dilution. Cultures are grown for 24 hr and cell-

[20] R. H. Schiestl and R. D. Gietz, *Curr. Genet.* **16**, 339 (1989).

[21] M. D. Rose, F. Winston, and P. Hieter, "Methods in Yeast Genetics." Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1990.

free medium is harvested from pooled cultures by centrifugation. Enzyme accumulates to greater than 50% of the total protein in the medium. medium is harvested from pooled cultures by centrifugation. Enzyme accumulates to greater than 50% of the total protein in the medium.

Day 6. Subsequent steps are carried out at 4°. Cell-free medium is diluted with 3 volumes of 40 m*M* Bis–Tris base and mixed with 20 ml Fast Flow Q-Sepharose (Pharmacia, Piscataway, NJ) per liter of culture medium. The resin is collected in a column, washed with one column volume of 40 m*M* Bis–Tris-Cl, pH 7.0, 2 m*M* $CaCl_2$, and eluted with a linear gradient (3 column volumes) of 0–0.5 *M* NaCl in the same buffer. The active peak is pooled and pressure dialyzed against NBC buffer (100 m*M* NaCl, 40 m*M* Bis–Tris-Cl, pH 7.0, 10 m*M* $CaCl_2$) using a YM30 membrane (Amicon, Danvers, MA). Because of the high expression of ss-Kex2 obtained with strain ABY01, the enzyme is ≥97% pure at this stage. An additional chromatography step on Mono Q (Pharmacia) as used in the original purification method may afford a higher degree of purity.[14]

1040 Medium. Per liter: 1.7 g yeast nitrogen base without amino acids or $(NH_4)_2SO_4$ (Difco), 1.32 g $(NH_4)_2SO_4$, 5 g NH_4Cl, 5 g vitamin assay casamino acids (Difco), 8.37 g [bis(2-hydroxyethyl)imino]tris(hydroxymethyl)methane (Bis–Tris) base, 20 g D-glucose, 0.12 g L-tryptophan, and 0.24 g adenine hydrochloride. The pH should be 7.2.[14]

Standard Kex2 Assay. The standard assay[14] is performed in 50 μl of 200 m*M* Bis–Tris-HCl (pH 7.0), 1 m*M* $CaCl_2$, 0.01% (v/v) Triton X-100, 0.5% (v/v) dimethyl sulfoxide, and 100 μ*M* *tert*-butoxycarbonyl-Gln-Arg-Arg-MCA (Peninsula Labs, Burlingame, CA). Enzyme (≤75 U) is added, reactions are incubated at 37° for 4 min, and are terminated on ice with 0.95 ml 125 m*M* $ZnSO_4$. 7-Amino-4-methylcoumarin (AMC) released is measured fluorimetrically relative to a standard (λ_{ex} 385 nm; λ_{min} 465 nm). One unit of Kex2 activity equals 1 pmol AMC released per min.[13]

Quantitation of Protein and Active Sites

For values of k_{cat} to be meaningful, the concentration of active enzyme must be known.[14] Further, the fraction of a purified enzyme preparation that is active is a measure of how native it is and how authentically it represents the cellular activity. The numerator of such a fraction is determined by active site titration. The denominator can be determined by measurement of total protein concentration or by amino acid analysis as described below.

Determining Molar Protein Concentration by Amino Acid Analysis

1. Approximately 2 μg of highly purified protein diluted into 1 ml of water is precipitated by the addition of 0.1 ml of 72% (w/v) trichloroacetic acid in a clean glass test tube. The dried pellet is resuspended in 0.1 ml

of 6 *N* HCl and protein is hydrolyzed during a 24-hr incubation at 110° *in vacuo*.

2. The hydrolyzate is resuspended with 0.5 nmol norleucine as an internal standard and loaded on a Beckman 7300 amino acid analyzer (Beckman Instruments, Palo Alto, CA) for ninhydrin quantitation of amino acid residues.

3. The picomole yields of 14 amino acids (naturally occurring amino acids except Gly, Met, Cys, Trp, Asn, and Gln) are converted to molarity by dividing by the initial volume. Molar yields are plotted against predicted occurrences of amino acids determined from the amino acid sequence of the protein. Occurrences of Asp and Glu are taken as the sum of Asp and Asn and the sum of Glu and Gln, respectively. The slope of the best-fit line through the origin equals the molar concentration of the polypeptide. We have found this method to give good agreement with values determined from measurement of total protein, e.g., by the BCA assay (Pierce, Rockford, IL).

Initial Burst Titration of Active Sites. Serine peptidase active sites may be determined by detection of a leaving group or product in the first turnover if a rate-limiting step occurs later in the reaction cycle than acylation.[21a] For instance, in the hydrolysis of *p*-nitrophenyl ethyl carbonate, chymotrypsin exhibited biphasic kinetics. One molar equivalent of *p*-nitrophenol was released rapidly, followed by a slow, steady-state accumulation of the alcohol leaving group.[22] Initial burst titrations have been performed with ester substrates that acylate rapidly but are hydrolyzed slowly. Surprisingly, burst kinetics have been observed with the best available amide substrate of Kex2, Ac-Pro-Met-Tyr-Lys-Arg-MCA. The kinetic and mechanistic basis of this reaction will be described elsewhere. The following method is adapted from Brenner and Fuller.[14]

1. A quenched-flow mixer is fitted with an enzyme syringe containing at least 10 pmol of ss-Kex2 protease per reaction loop volume and a substrate syringe containing 40 μM Ac-Pro-Met-Tyr-Lys-Arg-MCA; 0.75 *M* citric acid pH 3.0 is used as a quench.

2. Time points of 4, 8, 12, 16, 20, 100, 200, 300, 400, and 500 msec are taken in triplicate. Zero time points are created manually by adding enzyme to the quench solution before substrate is added. From 0 to 50 pmol of AMC is added to the zero time points to create a standard curve of product.

3. With the RQF mixer (KinTek, University Park, PA), longer time points contain more quenching solution. Prior to reading the fluorescence

[21a] C. G. Knight, this series.

[22] B. S. Hartley and B. A. Kilby, *Biochem. J.* **56**, 288 (1954).

of samples, time points are adjusted with the appropriate volume of quench to render them uniform.

4. Product fluorescence is plotted against time. A best-fit line of the data from later time points ($t \geq 20$ msec) is used to calculate the magnitude of the initial burst, i.e., the y intercept.

Active-site-directed irreversible inhibitors such as peptidyl chloromethanes offer an alternative approach to active site titration and examination of specificity.[15] Increasing concentrations of Pro-Nvl-Tyr-Lys-Arg-CH_2Cl and Phe-Ala-Lys-Arg-CH_2Cl were incubated with a known quantity of ss-Kex2 protease for 2 hr. Inhibited samples were then added to saturating substrate and assayed briefly. When activity remaining was plotted against the ratio of nominal inhibitor concentration to enzyme, 1.5 to 2 equivalents of the peptidyl chloromethanes were required to inactivate the enzyme. Because the enzyme concentration had been determined by protein assay, amino acid analysis, and initial burst assay, this titration was taken to adjust the true concentration of the inhibitors.[15] However, this assay, the full protocol of which is provided in the original literature, adds to the arsenal of techniques that may be used to assess the purity and the concentration of active ss-Kex2 protease.

Assays of Stability and Activity as Function of pH and Buffer System

Measurements of pH dependence of enzyme activity are usually intended to probe the pK_a values of functional groups. Titratable groups include not only catalytic groups in the enzyme but also moieties in both the enzyme and substrate that affect substrate binding. Unfortunately, variations in pH and the chemical nature of the buffers used can also have unanticipated effects on protein stability. "Activity" measured in different buffers not only reflects intrinsic activity at a given pH but also an inactivation rate specific for the buffer, pH, and temperature. To avoid this pitfall, it is advisable to determine the effects of buffers on enzyme stability before investing substantial effort in pH titration of enzyme activity.

Determination of Half-Times for Loss of Activity

1. Reaction premixes (0.4 ml) are assembled on ice with various buffers at 200 mM, plus 0.01% Triton X-100, 1 mM $CaCl_2$, and 1000 U of ss-Kex2 protease. Two 40-μl controls are withdrawn from each premix and mixed with 0.8 ml of 125 mM $ZnSO_4$ plus 10 μl of 500 μM Boc-Gln-Arg-Arg-MCA; 100 pmol of AMC is added to one sample from each buffer.

2. The premixes are incubated at 37°. At intervals ranging from 1 to 300 min, 40 μl of each premix are removed to 10 μl of 500 μM Boc-Gln-

Arg-Arg-MCA and incubated at 37° for 1 min to assay enzyme activity remaining. Reactions are terminated with 0.8 ml of 125 m*M* $ZnSO_4$.

3. AMC fluorescence of reactions is compared to that of nonincubated samples in corresponding buffers. At pH 7, in Bis–Tris, the first incubated samples should have liberated 125 pmol of AMC, corresponding to 2.5% of the substrate. Activity is plotted as a function of time of preincubation at 37° and fitted to a first-order decay curve, $y = \text{limit}[1 - \exp(-kt)]$. The half-time for loss of activity is $(\ln 2)/k$.

As can be seen from the measured half-times for loss of activity (Table I), measurements of pH dependence of enzyme activity must proceed with extreme caution. For the purposes of studying enzyme chemistry, buffer inactivation rates are uninteresting on their own. They are, however, essential to measure to optimize the standard assay and to evaluate the limitations of performing assays in different buffers. In the case of Kex2 protease, the pH dependencies of initial rates have been measured using brief end point incubations[14] and the pH dependence of k_{cat}/K_m was measured using continuous monitoring of product formation at low concentrations of substrate.[18] Once the effects of buffer and pH on enzyme stability have been controlled, Kex2 exhibits a relatively simple titration curve with a pK_a of ~5.7, indicative of titration of a single ionizable group, most likely the catalytic His-213.[14]

Cleavage of Purified α-Factor Precursor

Prepro-α-factor consists of an N-terminal signal peptide and hydrophilic leader, or "pro," segment (about 64 residues) followed by four

TABLE I
HALF-TIMES FOR LOSS OF INITIAL VELOCITY AT 37°

	Half-time (min) for buffer					
pH	Sodium acetate	Na-MES	Bis–Tris-Cl	Na-HEPES	Na-Bicine	Na-CHES
5.0	36					
5.5		5				
6.0		18				
6.5		17				
7.0			250	6		
7.5				22		
8.0				5		
8.5					44	
9.0					17	
9.5						2

copies, in tandem, of α-factor separated by spacers that contain sites for proteolytic maturation: the pair of basic residues, -Lys-Arg-, followed by two or three Glu-Ala or Asp-Ala dipeptides. The first Kex2 site (-Ser-Leu-Asp-Lys-Arg + Glu-Ala-) abuts the pro segment and thus differs from the remaining three sites (-Pro-Met-Tyr-Lys-Arg + Glu-Ala-), which occur between α-factor repeats. Reactions with these and related sequences have been investigated in the context of peptidyl-MCA substrates,[14] internally quenched fluorogenic peptide substrates,[17] and peptidyl chloromethanes.[15] However, to test to what degree presentation of these sites as part of a folded polypeptide affects their cleavage by Kex2 protease, pro-α-factor has been purified from recombinant bacterial cells and assayed with purified Kex2 protease.[23] Heterologous expression of pro-α-factor was required because, in wild-type yeast, the precursor is cleaved by Kex2 protease, and in *kex2* mutant cells, it becomes heterogeneously glycosylated. Thermal and chaotrope denaturation curves measured by circular dichroism and intrinsic tryptophan fluorescence indicate that pro-α-factor possesses substantial secondary and probably tertiary structure.[23]

Pro-α-factor processing presents a complex kinetic problem because cleavage at the four sites generates five products and as many as nine possible proteolytic intermediates. Using a combination of SDS–PAGE, Edman degradation, and amino acid analysis, it has been possible to demonstrate that ss-Kex2 cleaves pro-α-factor efficiently and only at the expected sites. At a concentration of 6 μM, α-factor precursor is cleaved to 50% completion in ~80 min and to apparent completion in 12 hr by 3 nM ss-Kex2 at 30° without further degradation of either the pro segment or the α-factor repeats.[23] The first and most abundant intermediates lack the C-terminal one or two repeats, suggesting that the C-terminal two cleavage sites are the most accessible in the folded pro-α-factor structure. An inhibition assay has been used to attempt to quantify the interaction of ss-Kex2 and purified pro-α-factor. Added pro-α-factor inhibits hydrolysis of peptidyl-MCA substrates competitively. However, the apparent K_i for pro-α-factor exhibited a striking dependence on pro-α-factor concentration. The lowest $K_{i,app}$, ~2 μM, was observed with 1 μM pro-α-factor, with the measured "constant" increasing at higher precursor concentrations. This may be the result of pro-α-factor aggregation in this concentration range, evidence for which has come from the behavior of pro-α-factor in chromatography and equilibrium analytical ultracentrifugation.[24] These results strongly underscore the importance of understanding the effects of precursor aggregation, thought to occur in the case of many prohor-

[23] A. Bevan, C. Brenner, and R. S. Fuller, unpublished data (1993).
[24] A. Bevan, T. Holzman, and R. S. Fuller, unpublished data (1993).

mones and neuropeptide precursors in the trans-Golgi network, on cleavage by processing enzymes.

Genetic Analysis of Substrate Specificity

To complement the biochemical analysis of substrate recognition, we have developed a genetic system for analyzing and manipulating the specificity of Kex2 protease.[25] Because Kex2 cleavage of pro-α-factor is required for yeast α cells to mate with a cells, we have been able to use yeast mating assays to measure the effects of mutations in a pro-α-factor cleavage site on Kex2 recognition. The yeast mating system provides sensitive bioassays for monitoring cleavage of pro-α-factor by Kex2 protease. The "halo" assay permits direct assessment of α-factor secretion as demonstrated by a zone of growth inhibition in a lawn of sensitive *MATa* cells.[2,26] The quantitative mating assay, although a less direct measurement of α-factor secretion, has enormous sensitivity, and a dynamic range of $\geq 10^6$.[27] Mating can also be scored in a qualitative fashion by a replica-plating assay (see next section), which can be used to screen for mutations in Kex2 that alter the specificity of the enzyme and permit cleavage of mutant forms of pro-α-factor.

The yeast strain constructed for this system, CB012,[25] is a *MATα* haploid, so that mating depends on production of α-factor. CB012 bears disruptions of the *KEX2* structural gene and the *MFα1* and *MFα2* genes, which encode the major and minor forms of prepro-α-factor. Therefore, the tester strain is sterile unless it carries two plasmids, one with a functional *KEX2* gene and the other with a functional α-factor precursor gene. Auxotrophic markers allow selection and maintenance of plasmids: *trp1* for the *KEX2* plasmid and *ura3* for the α-factor precursor plasmid. Additional auxotrophic markers permit the selection, on minimal yeast plates, of prototrophic diploids formed by mating of CB012 with a *MAT***a** tester strain.

For controlled expression and ease of mutagenesis, the *MFα1* gene has been placed under the control of the galactose-inducible, glucose-repressible *GAL1* promoter on the single-copy centromere plasmid pBM258.[10] The precursor gene was further modified by deleting three α-factor repeats, fusing the first Lys-Arg ↓ Glu-Ala-Glu-Ala processing site to the fourth repeat of mature α-factor. Mating of CB012 containing this plasmid depends entirely on cleavage of the single, remaining site, and

[25] C. Brenner, A. Bevan, and R. S. Fuller, unpublished data (1993).
[26] D. L. Julius, L. Blair, A. Brake, G. Sprague, and J. Thorner, *Cell (Cambridge, Mass.)* **32**, 839 (1983).
[27] L. H. Hartwell, *J. Cell Biol.* **85**, 811 (1980).

the effects of amino acid substitutions on both sides of this cleavage site can be monitored by qualitative or quantitative mating assays. Unique restriction sites upstream and downstream from the Kex2 cleavage site permit alteration of the cleavage site by cassette mutagenesis.[28] A number of substitutions have been made this way for the P2 Lys residue.[25] For these substitutions, the following order of cleavage preference was established: Lys-Arg = 1.0 ≫ (Thr-Arg > Pro-Arg > Ile-Arg > Asn-Arg) = 10^{-2}–10^{-4} ≫ (Phe-Arg, Leu-Arg) ≤ 4×10^{-6}, where the number indicates the quantitative mating efficiency relative to the normal Lys-Arg site. Mating by strains containing the Thr-Arg, Pro-Arg, Ile-Arg, and Asn-Arg substitutions, although feeble, depended on the presence of the *KEX2* plasmid. Phe-Arg and Leu-Arg substitutions resulted in mating at levels as low as with negative controls (no *KEX2* plasmid, no *MFα1* plasmid, and insertion of a translational termination codon in the P2 position), suggesting that these molecules were not cleaved at all. Strains containing the *MFα1-100* plasmid produced smaller α-factor halos compared to the wild-type *MATα* strain, as expected from the reduced mating efficiency. Halo assays failed to detect any α-factor secretion by strains containing *any* of the mutant precursor genes, suggesting that α-factor production was much less than 1% of wild type,[29] and dramatically demonstrating the greater sensitivity of the mating assay. Mutations in the *KEX2* gene that act as second-site suppressors of these α-factor precursor mutations are being sought by random mutagenesis.

One-Step Site-Directed Mutagenesis

Though the structural basis for substrate specificity can be approached with random mutagenesis and genetic selection, other aspects of Kex2 activity can be investigated using site-directed mutagenesis.[18] We have developed a rapid new method of site-directed mutagenesis that relies on the high efficiency of homologous recombination in mitotic yeast cells combined with the rapidity of the polymerase chain reaction (PCR).[30] We have applied this method, "one-step site-directed mutagenesis," to assess the effects of amino acid substitutions for the Kex2 oxyanion hole Asn-314 on the biosynthesis and activity of the enzyme.[18] The strategy is depicted in Fig. 2. Mutations are obtained in a yeast expression plasmid simply by transforming yeast with a linearized plasmid along with a PCR product primed with a mutagenic primer. In the mutagenesis of Asn-314,

[28] J. A. Wells, M. Vasser, and D. B. Powers, *Gene* **34**, 315 (1985).
[29] J. Kurjan, *Mol. Cell. Biol.* **5**, 787 (1985).
[30] R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich, *Science* **239**, 487 (1988).

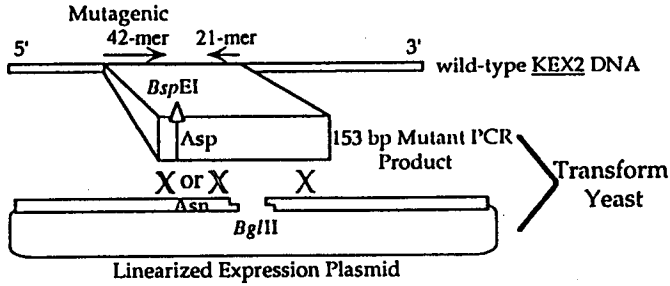

FIG. 2. One-step site-directed mutagenesis strategy. A 153-bp fragment encoding an amino acid substitution at codon 314 and silent substitutions at codon 312, which introduce a site for BspEI, was generated by PCR using the indicated primers. This fragment was cotransformed with a BglII digested yeast expression plasmid pG5-KEX2Δ613. Ligation of the linear plasmid or recombination on both sides of the BglII site is required for the plasmid to be replicated in yeast. On the left side of the BglII site, recombination to the left of the substitutions (distal), indicated by the X, generates a mutated plasmid and recombination to the right of the substitutions (proximal), indicated by X, generates wild-type plasmid.

transformants containing a plasmid with the desired substitutions of Ala and Asp for the oxyanion hole Asn-314 in Kex2 were identified by three methods: (i) assaying secreted Kex2 proteolytic activity, (ii) detecting a new restriction site in the plasmid-borne *KEX2* gene after PCR amplification, and (iii) performing qualitative mating assays.[18] Advantages of the method are that it requires no subcloning or passaging of constructs through *Escherichia coli* and that mutant proteins are immediately expressed in a yeast host strain and can therefore be purified and characterized without delay.

One-Step Site-Directed Mutagenesis of Kex2 Oxyanion Hole. As shown in Fig. 2, a 153-bp mutant PCR product that begins 5′ to Asn-314 and ends 3′ to *Bgl*II is generated from a wild-type *KEX2* template. The 5′ PCR primers are mutagenic 42-mers that introduce a silent *Bsp*EI site at codon 312 and change codon 314 from Asn to Ala or Asp at primer positions 25 to 32. This allows efficient priming by the 3′ ends of the primers and permits recombination distal (i.e., 5′) to the altered sites. The 3′ primer, a 21-mer, contains only wild-type sequences. To obtain recombinant plasmids, yeast strains containing a wild-type or a mutant chromosomal *KEX2* gene are transformed with the PCR product and pG5-*KEX2Δ613* plasmid DNA digested at *Bgl*II site 87 nucleotides 3′ to the Asn-314 codon. It is probably important to utilize a site for linearization in the target plasmid that is as close as possible to the site of mutation in order to increase the frequency of recovering mutant recombinants. Because 80% of the PCR product 5′ to the *Bgl*II site is 3′ to codon 314, most

recombinational transformants are expected to regenerate the wild-type plasmid. Consistent with this expectation, 15% of the transformants contain the desired mutations.[18]

Detection of One-Step Site-Directed Mutations by PCR

1. Analytical-scale PCR (25-μl reactions) contain 25 m*M* Na MES, pH 6.5, 50 m*M* KCl, 3 m*M* $MgCl_2$, 250 μM each deoxynucleoside triphosphate, 1 μM each primer, and 100 μg/ml bovine serum albumin (BSA); 1 μl of saturated yeast culture is added as a source of template DNA. Premixes are heated 7 min at 94°, 5 U *Taq* polymerase (Perkin-Elmer, Norwalk, CT) are added, and then reactions are performed in 30 cycles of 30 sec at 55°, 60 sec at 70°, and 30 sec at 94°. The primers employed are the nonmutagenic 3′ primer used to create the initial PCR product and a 21-mer primer corresponding to the nonmutagenic 5′ half of the original mutagenic 42-mers.

2. PCR products are digested with *Bsp*EI (New England Biolabs, Beverly, MA) and electrophoresed on a 2.5% agarose gel to determine whether the resulting PCR products contain the introduced mutations. As shown in Fig. 3, positive colonies obtained in strain ABY01 with an intact *KEX2* gene have a doublet after digestion. The upper band corresponds to the unaltered chromosomal *KEX2* gene. The lower band corresponds to the mutated gene, now rendered sensitive to *Bsp*EI. DNA sequence analysis proves that only the desired mutations have been introduced.[18]

Mating Assay for Presence of Site-Directed Mutations

1. A *MAT*α*kex2*Δ::*TRP1 leu2 trp1 ade2 his3 ura3* strain, CB017, is transformed with linearized pG5-*KEX2*Δ*613* plus Asn-314-Ala PCR product. Because of the low activity of these mutants, the site-directed mutant transformants are not expected to mate but the background of regenerated pG5-*KEX2*Δ*613* transformants are expected to mate.

2. CB017 transformants are grown on SDC-Ura plates, replica plated onto a lawn of ~5 × 10^5 DC14 cells (*MAT*a *his1*) on a YPD plate (rich,

FIG. 3. PCR assay for presence of site-directed mutations. *KEX2* DNA was amplified from 16 yeast transformants as described in the text, digested with *Bsp*EI, electrophoresed, and stained with ethidium bromide. Six lanes have doublets, indicating the presence of site-directed mutations.

nonselective medium[21]), incubated for 12 hr, and then replica plated onto an SD plate (minimal selective medium[21]), on which only diploids formed from mating of CB017 and DC14 cells can grow. A representative selective plate, photographed after 48 hr of incubation at 30°, is shown in Fig. 4.

Perspectives

Through the identification of the Kex2 protease as the authentic physiological pro-α-factor processing enzyme, yeast genetics and molecular biology have played a seminal role in the identification of the specific processing proteases likely responsible for maturation of a wide range of secretory precursors.[1-4] As a system, yeast continues to provide advantages in the study of kexin, the Kex2 protease. Rapid methods of site-directed and randomized mutagenesis combined with sensitive biological assays for enzyme activity and substrate specificity allow facile identification of mutant enzymes of significant interest, which can be overproduced and purified within a few days in quantities sufficient for both biochemical and structural analysis. In addition, the capability of purifying large amounts

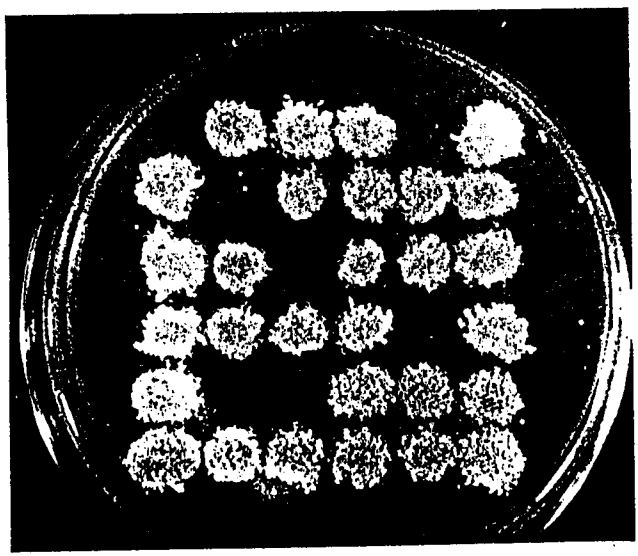

FIG. 4. Mating assay for presence of site-directed mutations. The CB017 transformant at the upper left contained plasmid pG5 (vector without *KEX2*), the next transformant to the right contained pG5-*KEX2Δ613*, and the other 34 were chosen at random from transformants of *Bgl*II-cleaved pG5-*KEX2Δ613* with the Asn[314]-Ala PCR product. Six out of the 34 transformants tested did not mate, indicating the presence of site-directed mutations.

of modified pro-α-factor produced through bacterial expression will permit quantitative biochemical analysis of the interactions between enzyme and substrate variants identified by *in vivo* assays. As a result, not only can Kex2 serve as a powerful model for the family of precursor processing proteases, but it also can stand alone as a powerful protein engineering system for studying catalysis and enzyme–substrate interactions.

Acknowledgments

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[12] Purification of Recombinant Soluble Forms of Furin Produced in Chinese Hamster Ovary Cells

By KAZUHISA NAKAYAMA

Introduction

The maturation of biologically active peptides and proteins often involves the limited proteolysis of larger precursors. In endocrine and neuronal cells, precursors of bioactive peptides are cleaved at paired basic amino acids within the regulated pathway of secretion.[1,2] By contrast, in nonneuroendocrine cells various secretory and membrane proteins are produced from precursors through cleavage at sites often marked by the consensus sequence, Arg-Xaa-(Lys or Arg)-Arg(RXK/RR), within the constitutive secretory pathway.[3] The regulated pathway is characteristic of endocrine and neuronal cells, and serves to store the peptides in secretory granules and to release them in response to external stimuli.[4] On the other hand, the constitutive pathway is present in all types of cells, and serves to release molecules continuously without storage.[4]

Research on processing endopeptidases has advanced with investigation of kexin, the Kex2 protease of the yeast *Saccharomyces cerevisiae*. It is a Ca^{2+}-dependent serine protease with a subtilisin-like catalytic domain and is involved in processing of pro-α-factor and pro-killer toxin at

[1] K. Docherty and D. F. Steiner, *Annu. Rev. Physiol.* **44**, 625 (1982).
[2] Y. P. Loh, M. J. Brownstein, and H. Gainer, *Annu. Rev. Neurosci.* **7**, 189 (1984).
[3] M. Hosaka, M. Nagahama, W.-S. Kim, T. Watanabe, K. Hatsuzawa, J. Ikemizu, K. Murakami, and K. Nakayama, *J. Biol. Chem.* **266**, 12127 (1991).
[4] T. L. Burgess and R. B. Kelly, *Annu. Rev. Cell Biol.* **3**, 243 (1987).