Characterization of a Vacuolar Protease in Neurospora crassa and the Use of Gene RIPing to Generate Protease-deficient Strains*

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We have isolated a gene from Neurospora crassa that appears to encode a pepstatin-sensitive protease found both in membranes and in soluble contents of vacuoles. The gene contains two introns and encodes a 396-residue protein with a molecular mass of 42,900 Da. Because of the similarity of the protein to proteinase A in Saccharomyces cerevisiae the gene has been named pep-4.

Strains with mutations in the pep-4 gene were generated in vivo by the gene RIPing procedure described by Selker and Garrett (Selker, E. U., and Garrett, P. W. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 6870–6874). The mutant strains were deficient in pepstatin-sensitive protease activity and did not appear to produce a major 42-kDa polypeptide in the vacuole. The mutant strains grew at the same rate as the wild type and had no other observable phenotype. When compared with inactivation of the PEP4 gene of S. cerevisiae, inactivation of the pep-4 gene in N. crassa produced a phenotype that was different in several ways. In N. crassa the mutant strains did not exhibit reduced sporulation or reduced viability after nitrogen starvation, and they had elevated levels of proteinase B and carboxypeptidase activities. The pep-4 gene appears to encode the N. crassa, homolog of proteinase A, but the maturation of vacuolar hydrolases appeared to be less dependent on this protease than has been observed in S. cerevisiae.

The lysosomal compartment of eucaryotic cells contains a large variety of hydrolytic enzymes. Most of these appear to be synthesized in the endoplasmic reticulum as inactive precursors, which are then modified in the Golgi and delivered to the lysosome. Activation occurs in the lysosome by the proteolytic removal of part of the polypeptide chain (1–5). In Saccharomyces cerevisiae proteinase A, the product of the PEP4 gene, has been shown to play a major role in the activation of vacuolar proteases. For example, cleavage by proteinase A activates proteinase B and carboxypeptidase Y. Proteinase B can, in turn, activate other hydrolases. Thus, proteinase A appears to be at the top of an activation cascade, and cells containing pep4 null mutations are deficient in at least three different proteases (6, 7). Proteinase A itself is made as a prepropeptidase. A 21-amino acid signal sequence is removed in the endoplasmic reticulum, and an additional 55 amino acids are cleaved autocatalytically in the vacuole to activate the enzyme.

We have been investigating the structure and function of vacuoles in Neurospora crassa (8). These organelles are very similar in size and composition to mammalian lysosomes. Because of the high density of vacuoles in N. crassa they can be isolated in good yield and purity. One of the most abundant proteins in the vacuole is a polypeptide of approximately 40 kDa (9). In our efforts to identify subunits of the vacular ATPase, we obtained evidence, described in this report, that the abundant 40-kDa protein is proteinase A. (We subsequently identified a 41-kDa subunit of the vacular ATPase that co-migrates in polyacrylamide gels with proteinase A (10).)

The characterization of proteinase A in N. crassa is important for several reasons. First, to our knowledge, this is the only protein in the vacular lumen to be identified in any filamentous fungus. (A gene encoding a serine protease that probably resides in the vacuole has been isolated from Aspergillus niger (11). However, direct information on the location of the gene product has not been reported.) If genes for several vacuolar proteases can be isolated, then the mechanisms of protein targeting to the vacuole can be investigated. Second, investigation of the structure and maturation of proteinase A from N. crassa will permit a test of hypotheses developed in S. cerevisiae. Much of our knowledge of vacuolar biogenesis is based on data obtained with yeast. It is important to see the extent to which these observations can be applied to other organisms. It should be noted that although S. cerevisiae and N. crassa are both fungi, the evolutionary distance between the yeasts and the filamentous fungi is nearly as great as the distance between yeasts and animals (12). Third, the identification of the gene for proteinase A makes it possible to generate protease-deficient strains. As mentioned above some pep4 strains of S. cerevisiae are deficient in multiple proteases. They have normal growth rates and have become widely used laboratory strains. Because of the industrial importance of protein production in filamentous fungi, protease-deficient strains could be very useful.

EXPERIMENTAL PROCEDURES

Materials—Oligonucleotides were purchased from DNA International (Eugene, OR), and hide powder azure protease substrate was purchased from Calbiochem. The peptide N-[Suc-cyclo(L-argyl-L-prolyl-L-phenylalanyl-L-histidyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 7-amido-4-methylcoumarin, N-benzyo-L-tyrosine p-nitroanilide protease substrate, pepstatin, and leucine aminopeptidase (L5658) were purchased from Sigma.

Preparation and Sequencing of Vacuolar Proteins—Vacuoles were prepared as described (13, 14). Vacuoles were lysed by suspension in 1 mL EGTA, adjusted to pH 7.5 with Tris, and centrifuged at 20,000 × g for 5 min to remove high density contaminants, mostly cell wall fragments. The supernatant was centrifuged at 150,000 × g for 10 min. The final supernatant was kept as the “vacuolar contents” fraction, while the pellet contained the vacuolar membranes. Membranes were resuspended in 1 mL EGTA, pH 7.5, at a concentration of approximately 3 mg protein/mL. For some experiments the membrane sector of the vacuolar ATPase was prepared as described (9). Both vacuolar membranes and contents were stored at −80 °C.

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Vacuolar protease in N. crassa

Vacuolar proteins were further purified by polyacrylamide gel electrophoresis. Tryptic fragments of the proteins were isolated and purified as described (15). To summarize briefly, individual bands were excised from polyacrylamide gels, electrodried, precipitated with acetone, redissolved in 2 M urea, 0.1 M NH₄CO₃, pH 7.5, and digested with trypsin (0.1 μg, 24 h, 37 °C). The tryptic fragments were purified by high pressure liquid chromatography, using a C18 column (Vydac 218TP). Peptides were sequenced in our laboratory using a Perkin-Elmer 2090CE integrated microsequencing system.

Isolation and Sequencing of the pep-4 Gene—The polymerase chain reaction was used to amplify a portion of the PEP4 gene (see Fig. 1). The 5′ primer, containing an EcoRI restriction site, was GAAGTTCAAGC-CTG/T/C/TG/GAA/C/T/GAG/GA/GGATC/CC. The 3′ primer, containing a HindIII restriction site, was the antisense of GG(T/C)/AAT/C/TG/C/TG/GA/C/T/GAG/G. Using N. crassa genomic DNA we amplified a 3.0-kb fragment, which was the size used to screen a cDNA library in the ZAP vector. Two DNA clones, 1.1 and 1.7 kb, were isolated. The ends of the shorter cDNA, all of the longer cDNA, and the genomic DNA described below, were sequenced (16), using the Cyclone kit (International Biotechnologies, New Haven, CT), to obtain a series of single stranded plasmids with nested deletions. The 3′ ends of both cDNA clones were polyadenylated. The longer cDNA clone was used to screen the genomic library in the wild type pSV50 (vector) (17). Two positive cosmids were isolated, 6.5-A and 31.5-E. By using the 1.7-kb cDNA of proteinase A to probe 32P-digested genomic DNA, the pep-4 gene was found to lie within two adjacent PsiI fragments. Each of these fragments was subcloned into the Bluescript SK− vector (Stratagene, La Jolla, CA). The smaller fragment (0.6 kb) was excised from polyacrylamide gels, electroeluted, precipitated with acetone, redissolved in 2 M urea, 0.1 M NH₄CO₃, pH 7.5, and digested with trypsin (0.1 μg, 24 h, 37 °C). The tryptic fragments were purified by high pressure liquid chromatography, using a C18 column (Vydac 218TP). Peptides were sequenced in our laboratory using a Perkin-Elmer 2090CE integrated microsequencing system.

RESULTS

Isolation of the pep-4 Gene—The genomic fragment with the integrated plasmid was not closely linked to the wild-type control, and six had 10–20% of the activity of wild type.

To determine if the pep-4 gene was indeed mutated, the P6-45 strain was grown in vacuoles and compared with the wild-type strain. This mutant had lost the ability to recognize and digest the pep-4 gene. The P6-45 strain was also isolated by complementation of the pep-4 gene. Two additional alleles, an additional 15 gag-prepeptides were isolated from the original P6 × 74a cross. Two of these with low proteinase A activity, designated pep-4-47 and pep-4-49 were used for all experiments described in this report. To analyze the mutations in these strains, genomic DNA was prepared and sequencing was achieved by the polymerase chain reaction. The positions and sequence of the primers are shown in Fig. 1. The amplified DNA was separated on an agarose gel, purified by the GeneClean procedure (Bio 101, La Jolla CA), and subcloned into the pTRI blue vector (Novagen, Madison, WI). Single-stranded DNA was prepared, essentially by the procedure recommended by the manufacturer. The ends of the mutated pep-4 genes were sequenced, further primers were made, and the process was repeated until the entire amplified region had been sequenced.

Protease Assays—N. crassa cells were grown 3–5 days at 25 °C, in 20-ml stationary liquid cultures of Vogel's minimal medium as described (24). Preliminary experiments showed that protease activity was essentially the same after 3, 4, or 5 days of growth. Cells were harvested by filtration through Whatman No. 1 filter paper, rinsed in 0.1 M Tris, pH 7.5, suspended in approximately 10 ml of 0.1 M Tris, pH 7.5, and disrupted in a Beadbeater homogenizer (Biospec Products, Bartlesville, OK) for 1 min in an ice-jacketed chamber. The homogenate was transferred to a 12-ml tube and centrifuged for 10 min at 3000 rpm in a Sorvall SS34 rotor to pellet heavy debris and intact cells. The supernatant was transferred to 1.5-ml microtubes and centrifuged for 20 min at 18,000 × g. The final supernatant was removed with a pipette and placed on ice. For most protease assays the cell extract was used within 1 h of preparation, but the extract also retained significant activity if frozen in liquid nitrogen and stored at −80 °C. Proteinase A and proteinase B were assayed as described (2), except that the hydrolysis of the peptide N-succinyl-γ-arginyl-γ-prolyl-L-phenylalanyl-L-histidyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine 7-amido-4-methylcoumarin was carried out at 37 °C. Protein concentration of the cell extracts was determined by the method of Lowry et al. (25).

The sequence of the genomic DNA is shown in Fig. 1. By comparing cDNA and genomic DNA we identified two short introns of 63 and 71 base pairs. As is typical for many N. crassa genes, the introns occur early in the protein coding region (26). Also typical was the observation that the cDNAs were polyadenylated at different sites, in this case separated by 67 base pairs. The chromosomal position of the gene was precisely determined by the fortuitous observation that it was present in two cosmid clones that had been previously mapped. Based on the data of Maclung et al. (27) the gene lies on linkage group VII in the middle of a 25-kb region that separates the frq and for genes.

Analysis of the open reading frame showed that the gene encoded a protein with 396 amino acids, 42,900 daltons. When aligned with proteinase A from S. cerevisiae (Fig. 2) 57% of the residues were identical. Proteinase A is an aspartyl protease that has two highly conserved active sites (1, 6, 7). These regions, boxed in Fig. 2, are essentially identical in S. cerevisiae and N. crassa. Also identical are the two putative glycosylation sites and two suggested sites of disulfide bond formation (Cys116–Cys121 and Cys318–Cys351). These data strongly suggested that the gene we had cloned encoded the N. crassa homolog of proteinase A; thus, it was named pep-4.

In S. cerevisiae proteinase A is synthesized as a large inactive precursor. The N terminus of the protein is cleaved, first in
the endoplasmic reticulum and second in the vacuole to generate the active, mature form of the protein. One of the tryptic peptides that we sequenced corresponded to residues 71–90 in the N. crassa protein (underlined in Fig. 2). This peptide was particularly interesting because it was not preceded in the sequence by an arginine or lysine residue as would be predicted for trypsin cleavage. Furthermore, the beginning of this peptide corresponds precisely to the beginning of the mature form of proteinase A from S. cerevisiae. The N. crassa peptide may have been derived from the N terminus of the mature protein. Comparison of "presequences" in proteinase A is discussed further below.

Inactivation of the pep-4 Gene—To inactivate the pep-4 gene we took advantage of the RIPing phenomenon (21, 22). As described under "Experimental Procedures," we introduced an extracopy of the pep-4 gene into wild type cells by transformation. The transformed cells were then put through a sexual cycle. In N. crassa, haploid cells of different mating type fuse and undergo several rounds of nuclear division before nuclear fusion occurs. In these premeiotic cells, nuclei with two copies of a given gene are subject to a poorly understood process whereby duplicated DNA becomes mutated. GC base pairs are converted to AT base pairs in both the introduced and the endogenous copy of the gene. 5–50% of nuclei with duplicated DNA are affected, and the number of point mutations varies from 1 to more than 50 per kb of DNA (22).

The transformed strains contained an extra copy of a 1.8-kb PstI/HindIII fragment of genomic DNA, which included the entire protein coding region of the pep-4 gene. Haploid progeny of a cross between transformant and wild type were examined.

**Fig. 1.** Sequence of the pep-4 gene. Introns within the pep-4 gene are in lowercase letters. The regions corresponding to two polyadenylated cDNAs are indicated by arrows. The positions of four primers are shown in boxes. Two primers within the protein coding region were used in the initial polymerase chain reaction as described under "Experimental Procedures." The primers at the 3' and 5' ends were used to amplify the gene in pep-4 mutant strains.

**Fig. 2.** Alignment of amino acid sequences of proteinase A. The top sequence (S.c.) is from S. cerevisiae (6, 7). The lower sequence (N.c.) is from N. crassa (this report, GenBank accession number U36471). The N terminus of the mature S. cerevisiae protein is indicated by the arrow. The sequence of a tryptic peptide from the N. crassa protein, presumably at the N terminus, is underlined. Residues at the active site of the enzyme are shown in the two larger boxes. Putative glycosylation sites are in two smaller boxes. Amino acids identical in both sequences are marked with an asterisk.

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for changes in NlaIII restriction sites within the pep-4 gene or for loss of protease activity in cell extracts (see "Experimental Procedures"). Two of the progeny, designated pep-4–47 and pep-4–49 were selected for further analysis. Using the polymerase chain reaction, genomic DNA corresponding to the entire protein coding region of the pep-4 gene was amplified. The 5' primer was 30 base pairs upstream of the PstI site that marked the end of the duplicated region, thus ensuring that the amplified fragment corresponded to the endogenous copy of the pep-4 gene. In addition, the introduced, extra copy of pep-4 had apparently integrated at a site not closely linked to the pep-4 locus, because the introduced and endogenous pep-4 genes segregated independently (data not shown). The amplified DNA was sequenced as described under "Experimental Procedures" and found to be mutated at many sites. In strain pep-4–47, 99 nucleotide changes were found, scattered throughout the length of the duplicated region. In strain pep-4–49, 22 point mutations were detected, all but two in the 5' half of the duplicated region. The changes were typical of those observed previously in RIPed genes, GC to AT transitions with cytosine being the most common target.

The effect of these mutations on the protein sequence is shown in Fig. 3. In strain pep-4–49 all changes were missense mutations. Fourteen nucleotide changes were silent, while the rest were predicted to change seven amino acids, all clustered in the region of residues 145–202. In the more heavily mutated strain, pep-4–47, a stop codon was introduced after amino acid 31. Furthermore, the highly conserved G in the 5' splice site of the first intron was mutated.

Analysis of Protease Activity in Strains with Mutations in the pep-4 Gene—We used whole-cell extracts to assay the hydrolysis of acid-denatured hemoglobin (see "Experimental Procedures"). In S. cerevisiae protease A has been reported to be the only protease to catalyze the reaction at acid pH (2). We also tested the effect of pepstatin, a specific inhibitor of aspartyl proteases. As shown in Fig. 4, extract from the wild type cells had significant levels of protease activity (11 μg of tyrosine/ min/mg), which was inhibited 85% by pepstatin. The optimum pH for this activity was 3.2, the same as reported for S. cerevisiae (30). Protease activity in the mutant strains, pep-4–47 and pep-4–49, was approximately 15% the level observed in the wild-type and was not significantly inhibited by pepstatin. Our interpretation of these data was that the mutant strains had no measurable protease A-like activity and that the pepstatin-resistant activity came from other enzymes.

Protease A has also been reported to hydrolyze the octapeptide \( N\text{-sucinyl-L-arginyl-L-prolyl-L-phenylalanyl-L-histidyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine} \) 7-amido-4-methylcoumarin (2). As shown in Fig. 5, extract from wild type N. crassa hydrolyzed the peptide at significant rates, and the hydrolysis was inhibited 63% by pepstatin. The pep-4 mutant strain had only 36% of the activity of the wild type, and this activity was not significantly inhibited by pepstatin. Thus, the mutant strain appeared to be deficient in all of the protease A-like activity as assayed by hydrolysis of this peptide.

We also measured the hydrolysis of hide powder azure, diagnostic of protease B activity, and of \( N\text{-benzoyl-L-tyrosine} \) p-nitroanilide, diagnostic of carboxypeptidase activity (2), as shown in Table I. The mutant strains had significant levels of both types of activity. These experiments were repeated several times; the mutant strains consistently yielded 1.5-2.0-fold higher activity than the wild type.

Analysis of the pep-4 Protein—Vacuoles were prepared from the two mutant strains and from the wild type. The vacuoles were lysed by suspension in 1 mM EGTA. Membranes and soluble contents were separated by centrifugation and analyzed by gel electrophoresis. As shown in Fig. 6 an abundant polyepitope of 40 kDa, presumably protease A, was observed in the wild type but not in either of the mutant strains. The pattern of polypeptides was clearly different for the membranes versus the soluble contents, however protease A was a major component of both.

The proteins from vacuolar membranes and contents were transferred to a nylon membrane and incubated with a polyclonal antibody, raised against the protease A from S. cerevisiae. The pattern of stained bands was the same for vacuoles from wild-type and from strain pep-4–49.
from the wild type. Containing ammonium nitrate to test for the resumption of growth.

were rinsed and resuspended in normal Vogel's medium containing 0.05% yeast nitrogen limited medium (data not shown). Also, in contrast to pep-4 mutant strains of S. cerevisiae (28), mutations in the N. crassa pep-4 gene did not significantly affect fertility or viability after nitrogen starvation. A cross between pep-4-47 and pep-4-49 produced abundant viable spores. To test the effect of the pep-4 mutation on the survival of cells after nitrogen starvation, 3-day-old cultures of both pep-4 mutant strains and the wild-type strain were transferred to nitrogen-free Vogel's medium. The cultures were incubated at 25 °C. After 2, 3, 4, or 5 days the cultures were rinsed and resuspended in normal Vogel's medium containing ammonium nitrate to test for the resumption of growth. All cultures grew well, and mutants were indistinguishable from the wild type.

**DISCUSSION**

We have isolated a gene that appears to encode the N. crassa homolog of proteinase A. The high degree of sequence conservation at the putative active site and sensitivity of the pep-4 gene product to inhibition by pepstatin argues strongly that the enzyme is an aspartyl protease. The pep-4 gene product shares approximately 30% sequence identity with mammalian cathepsins, pepsinogens, and renins but is 57% identical to proteinase A from S. cerevisiae. Antibody raised to the yeast proteinase A cross-reacts with the N. crassa polypeptide, the protease was located in the vacuole, and the specificity for substrates, measured in vitro, was the same as reported for yeast proteinase A.

Proteinase A in S. cerevisiae is made as a propeptide that undergoes maturation by cleavages in the endoplasmic reticulum and in the vacuole (3, 6, 7). We do not have direct evidence for processing of the N. crassa protease, but one of the tryptic peptides we sequenced was not preceded by the expected lysine or arginine residue. It is likely that this peptide was derived from the N terminus of the mature protein, indicating that the N. crassa and S. cerevisiae precursor proteins are cleaved at the same position. The 20 amino acids preceding the cleavage site are completely dissimilar, but the amino acids at the N terminus of the mature protein are very similar. A recent analysis of the propeptide of proteinase A from S. cerevisiae identified 39 functional mutant forms containing random sequence in the region preceding the cleavage site (29). A single residue, lysine at position 53, was identified as important for processing. This lysine is present in the N. crassa propeptide, part of a sequence of six amino acids (HLGQK) identical in both yeast and N. crassa propeptides. Conceivably, the region containing these six amino acids could interact with conserved regions in the mature protein to determine the site of cleavage.

Our data indicated that in N. crassa proteinase A is one of the most abundant proteins in the vacuole. In S. cerevisiae the purified enzyme behaves like a typical soluble protein (30), and in N. crassa most of the enzyme was found in the soluble contents. However, it was surprising to observe that proteinase A was also the most prominent polypeptide in vacuolar membranes. Because of our interest in other proteins in the vacuolar membrane, particularly the proton-pumping ATPase, we wished to generate a strain of N. crassa that lacked proteinase A. We used the RIPing phenomenon, which appears to be unique to N. crassa (22) to inactive the pep-4 gene. Seven amino acids were changed in one mutant strain, and a stop codon was introduced early in the protein coding region of a second mutant strain. Cell extracts from the pep-4 mutant strains lacked essentially all proteinase-A activity as assessed by hydrolysis of two different substrates. Neither mutant strain had detectable proteinase A polypeptide in the vacuole,
as assayed by gel electrophoresis or by the Western blotting procedure. In the strain with only missense mutations (pep-4-49) the polypeptide must be quickly degraded or not targeted to the vacuole. Inactivation of the pep-4 gene had no measurable effect on growth rate or viability of the strains.

pep-4 mutants of N. crassa differ from pep4 mutants of S. cerevisiae in two significant ways. First, in S. cerevisiae a/a diploids that are homozygous for pep4 are sporulation-deficient (28). In N. crassa, crosses between two pep-4 mutant strains produced a normal yield of viable spores. Secondly, pep4 mutants of S. cerevisiae are deficient in several vacuolar proteases, including protease B and carboxypeptidase Y (6, 7). In N. crassa these proteases are not deficient in pep-4 mutants. In fact the activities measured were higher than in the wild type strain, as if the loss of proteinase A was compensated by higher levels of other proteases. The multiple protease deficiency in yeast pep4 strains has been explained by the hypothesis that proteinase A is needed for the proteolytic maturation of several other proteases. The requirement does not appear to be absolute, however, because there may be two or more enzymes that can cleave and activate precursor proteins for vacuolar hydrodases. Woolford et al. (31), in a recent study of proteinase A mutants, concluded that “a great deal of redundancy has been built into the system.” Our analysis of pep-4 mutants in N. crassa supports this conclusion.

Although the pep-4 mutant strains we have generated retain significant protease activity, they may still be useful for the isolation of proteolytically sensitive proteins. Proteinase A appears to be very abundant in the wild type strains, and a significant amount is tightly associated with the vacuolar membrane. In studies of the vacuolar ATPase we have found that some of the subunits (e.g. the 57- and 98-kDa polypeptides) are particularly sensitive to proteolysis (9). Use of the pep-4 strain for purification of the ATPase and other proteins of the vacuolar membrane may provide a greater yield of undamaged polypeptides.

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