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Identification of the Structural Gene for Dipeptidyl Aminopeptidase yscV (*DAP2*) of *Saccharomyces cerevisiae*

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Mutants of *Saccharomyces cerevisiae* lacking dipeptidyl aminopeptidase yscV were isolated from a strain already defective in dipeptidyl aminopeptidase yscIV, an enzyme with overlapping substrate specificity. The mutants were identified by a staining technique with the chromogenic substrate Ala-Pro-4-methoxy-β-naphthylamide to screen colonies for the absence of the enzyme. One of the mutants had a thermolabile activity, indicating that it contained a structural gene mutation. The 53 mutants analyzed fell into one complementation group that corresponded to the yscV structural gene, *DAP2*. The defect segregated 2:2 in meiotic tetrads, indicating a single chromosomal gene mutation, which was shown to be recessive. Diploids heterozygous for *DAP2* displayed gene dosage effects with respect to yscV enzyme activity. The absence of dipeptidyl aminopeptidase yscV or the combined loss of both dipeptidyl aminopeptidases yscIV and yscV did not affect mitotic growth under rich or poor growth conditions. In contrast to the dipeptidyl aminopeptidase yscIV lesion (*ste13*), which leads to α sterility because strains secrete incompletely processed forms of the α-factor pheromone, the dipeptidyl aminopeptidase yscV lesion did not affect mating, and strains produced fully active α-factor pheromone. *dap2* mutants did not show any obvious phenotype under a variety of conditions tested.

A variety of proteinases have been detected in the yeast *Saccharomyces cerevisiae*, and their vital importance in cellular control has become apparent (for recent reviews, see references 4, 17, 35, and 36).

The unambiguous assignment of a proteinase to its intracellular function has only been possible by combining biochemical and genetic approaches as well as molecular biological techniques, for which yeast is most suitable. Thus, the implication of proteinase yscA (6, 18, 23a, 24, 41), proteinase yscB (37, 43; Mechler et al., in press), carboxypeptidase yscY (38), carboxypeptidase yscS (38), aminopeptidase yscI (33), aminopeptidase yscII (H. Hirsch, P. Suárez Rendueles, T. Achstetter, and D. H. Wolf, manuscript in preparation), dipeptidyl aminopeptidase yscIV (19), and proteinase yscF (3, 20) in processes of differentiation (proteinases ycsA, yscB, and yscF and carboxypeptidases yscY and yscS), general protein degradation during nitrogen starvation (proteinases yscA and yscB), metabolism of exogenously supplied peptides (carboxypeptidases yscY and yscS, aminopeptidase yscII), or processing of inactive precursors (proteinases yscA, yscB, and yscF, dipeptidyl aminopeptidase yscIV) has only been possible by using mutants devoid of these enzymes or by cloning their genes.

We have previously described a membrane-bound X-prolyl dipeptidyl aminopeptidase activity in yeast (14, 32), later reported to be due to at least two different enzymes (19). One of the enzymes is heat stable and was called dipeptidyl aminopeptidase yscIV (also called dipeptidyl aminopeptidase A; for nomenclature see references 1 and 4). The enzyme is coded for by the *STE13* gene and has been shown to be involved in α-factor precursor processing by removing dipeptides from the N-terminus of the pheromone precursor (19). Mutants lacking dipeptidyl aminopeptidase yscIV (*ste13*) are sterile when their mating type is *MAT*α (19, 29). The other enzyme is heat labile and was called dipeptidyl aminopeptidase yscV. The enzyme is bound to the vacuolar membrane (7) and has been purified and characterized as a serine peptidase exhibiting a very strong preference for substrates containing a penultimate proline residue (12).

This paper reports the isolation of mutants carrying lesions that lead to a temperature-sensitive dipeptidyl aminopeptidase yscV activity or to the absence of this enzyme activity. Our studies indicate that the gene designated *DAP2* is the structural gene for dipeptidyl aminopeptidase yscV. The properties of mutants lacking this enzyme as well as double mutants lacking both dipeptidyl aminopeptidase yscIV and yscV have been studied.

MATERIALS AND METHODS

Chemicals. Yeast extract, peptone, yeast nitrogen base, and agar were from Difco (Roth, Karlsruhe, Federal Republic of Germany [FRG]). Ethylmethane sulfonate was from Serva (Heidelberg, FRG). Ala-Pro-4-methoxy-β-naphthylamide, Ala-Pro-4-nitroanilide, and α-factor were supplied by Bachem (Bubendorf, Switzerland). Fast garnet GBC BF_4 salt was from Serva (Heidelberg, FRG). Octyl-β-D-glucopyranoside was supplied by Sigma (Taufkirchen, FRG). All other chemicals, which were of the highest purity available, were purchased from either Merck (Darmstadt, FRG) or Roth (Karlsruhe, FRG). All amino acids used were of the L-configuration.

Yeast strains. The genotype and source of the *S. cerevisiae* strains used in this paper are listed in Table 1.

Media and growth conditions. The general use and composition of all media have been described previously (24). Growth of cells was performed either in liquid complete medium (YPD; 1% yeast extract, 2% peptone, 2% glucose), rich medium (GNA; 5% glucose, 3% nutrient broth, 1%

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TABLE 1. Strains used

Strain	Genotype	Source or reference
X2180-1A	*MATa*	Yeast Genetic Stock Center
A2S3	*MATα ste13-1 ade6 leu1 trp5 his6 met1 can1 rme1 gal2*	29
RC629	*MATa sst1-2 ade2 ura1 his6 met1 cyh2 rme*	9
RC631	*MATa sst2-1 ade2 ura1 his6 met1 cyh2 rme*	9
ABYS60	*MATa pra1-1 prb1-1 prc1-1 cps1-3 ade2 his*	B. Mechler and D. H. Wolf, unpublished
PS3	*MATα ste13-1 prb1-1 prc1-1 cps1-3 ade2 his*	This work; segregant from cross A2S3 × ABYS60
PSA26	*MATα ste13-1 prb1-1 prc1-1 cps1-3 ape2-1 leu2 his*	This work; segregant from cross PS3 × AP-109
AP-109	*MATa prc1-1 cps1-3 ape2-1 leu2*	H. H. Hirsch and D. H. Wolf, unpublished
AP-116	*MATa prc1-1 cps1-3 ape2-1 leu2 lys2*	H. H. Hirsch and D. H. Wolf, unpublished
DPS-14	*MATα ste13-1 dap2-1 prb1-1 prc1-1 cps1-3 ape2-1 leu2 his*	This work (EMS mutagenesis of strain PSA26)
DPS-148	*MATα ste13-1 dap2-2 ade6 leu1 trp5 his6 met1 can1 rme1 gal2*	This work (EMS mutagenesis of strain A2S3)
DPSI		Diploid (DPS-14 × AP-116)
DPSI-4A	*MATa ste13-1 dap2-1 prb1-1 prc1-1 cps1-3 ape2-1 leu2 lys2 his*	This work; segregant from cross DPS-14 × AP-116
DPSI-4B	*MATa ste13-1 dap2-1 prc1-1 cps1-3 ape2-1 leu2 lys2*	This work; segregant from cross DPS-14 × AP-116
DPSI-8A	*MATa ste13-1 prb1-1 prc1-1 cps1-3 ape2-1 leu2*	This work; segregant from cross DPS-14 × AP-116
DPSI-8B	*MATα dap2-1 prc1-1 cps1-3 ape2-1 leu2 his*	This work; segregant from cross DPS-14 × AP-116
DPSI-8D	*MATa ste13-1 dap2-1 prb1-1 prc1-1 cps1-3 ape2-1 leu2 lys2 his*	This work; segregant from cross DPS-14 × AP-116
DPSI-9A	*MATa ste13-1 dap2-1 prc1-1 cps1-3 ape2-1 leu2 lys2 his*	This work; segregant from cross DPS-14 × AP-116
DPSI-12A	*MATα ste13-1 prc1-1 cps1-3 ape2-1 leu2 lys2 his*	This work; segregant from cross DPS-14 × AP-116
DPSII		Diploid (DPSI-8B × X2180-1A)
DPSII-2B	*MATα dap2-1 leu2 his*	This work; segregant from cross DPSI-8B × X2180-1A
DPSII-8B	*MATa dap2-1 prc1-1 cps1-3 ape2-1 his*	This work; segregant from cross DPSI-8B × X2180-1A
DPSII-18A	*MATα dap2-1 prc1-1 cps1-3 ape2-1 his*	This work; segregant from cross DPSI-8B × X2180-1A
DPSIII		Diploid (DPSI-4A × A2S3)
DPSIII-18B	*MATα ste13-1 dap2-1 prc1-1 cps1-3 ape2-1 ade6 leu his*	This work; segregant from cross DPSI-4A × A2S3
DPSIII-22C	*MATa ste13-1 prc1-1 cps1-3 ape2-1 ade6 leu his*	This work; segregant from cross DPSI-4A × A2S3
DPSIV		Diploid (DPSI-8A × DPS-148)
DPSIV-2A	*MATa ste13-1 dap2-2 prc1-1 met1*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-2B	*MATα ste13-1 dap2-2 prb1-1 cps1-3 trp5*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-4A	*MATa ste13-1 dap2-2 prb1-1 prc1-1 trp5 leu met1*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-4B	*MATα ste13-1 dap2-2 prb1-1 ade6 met1*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-4C	*MATa ste13-1 cps1-3 trp5 his6 ade6 leu*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-4D	*MATα ste13-1 prc1-1 cps1-3 his6*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-8A	*MATa ste13-1 prc1-1 ade6 leu met1*	This work; segregant from cross DPSI-8A × DPS-148
DPSIV-8D	*MATα ste13-1 prb1-1 prc1-1 cps1-3 trp5 his6 leu*	This work; segregant from cross DPSI-8A × DPS-148
DPSV		Diploid (DPSIV-8A × DPSIV-8D)
DPSVI		Diploid (DPSIV-2A × DPSIV-8D)
DPSVII		Diploid (DPSIV-2A × DPSIV-2B)
DPSVIII		Diploid (DPSIV-2A × DPS-14)
DPSIX		Diploid (DPSIII-22C × DPSI-12A)
DPSX		Diploid (A2S3 × DPSI-8D)
DPSXI		Diploid (DPSI-4B × DPSIII-18B)
DPSXII		Diploid (DPSII-8B × DPSII-18A)
DPSXIII		Diploid (DPSI-4B × DPSII-2B)
DPSXIV		Diploid (DPSI-9A × DPSIII-18B)

yeast extract), or mineral medium (MV; 0.67% yeast nitrogen base without amino acids, 2% glucose). When solid media were used, 2% agar was included. When necessary, media were supplemented with the amino acids required by auxotrophic strains. Mating between sterile strains (8) was induced by addition of 4 μg of synthetic α-factor per ml of medium. Spores were tested for secretion of normal α-factor on a lawn of strain RC629 and for incompletely processed forms of α-factor on a lawn of strain RC631 (19) by the method of Manney et al. (22) with YPD medium plates buffered to pH 5.0 with citrate phosphate. Mating of haploid strains was performed on YPD medium buffered to pH 5.0 with 1 M citric acid. Sporulation of diploid strains was induced by a medium containing 1% potassium acetate, 0.125% yeast extract, 0.05% glucose, and 2% agar. The same medium without agar was used as the liquid sporulation medium. When cells to be used for biochemical experiments were grown on solid medium, half of a petri dish was inoculated per strain. After 48 h of growth at 23 or 30°C, cells were harvested and extracts were prepared.

Preparation of extracts. Cells were harvested from solid YPD medium and suspended in 200 μl of 0.1 M Tris chloride buffer, pH 7.0; 200 μl of acid-washed glass beads (0.5 mm diameter) were added, and the mixture was vigorously shaken on a Vortex shaker for seven periods of 1 min each with 1-min intervals of cooling in ice. The crude extracts were carefully removed from the glass beads and used for enzyme assays.

Enzyme assays. In strains carrying dipeptidyl aminopeptidases yscIV and yscV, total dipeptidyl aminopeptidase activity was determined with Ala-Pro-4-nitroanilide as the substrate as described previously (32). Dipeptidyl aminopeptidase yscIV and yscV activities were distinguished by their difference in heat stability. Heating of crude extracts at 60°C for 20 min readily inactivates dipeptidyl aminopeptidase yscV almost completely, while dipeptidyl aminopeptidase yscIV is stable under those conditions, without significant loss of activity (7, 19). When the activity of enzyme preparations of strains carrying a thermolabile dipeptidyl aminopeptidase yscV activity were measured, assays were

TABLE 2. Dipeptidyl aminopeptidase levels in crude extracts of segregants of the cross of strains DPS-14 (*ste13-1 dap2-1*) × AP-116 (*STE13 DAP2*)[a]

Tetrad	Spore	Dipeptidyl aminopeptidase activity (mU/mg)		% Activity remaining after heating	Genotype
		No preincubation	Preincubation (60°C, 30 min)		
4	A	0.03	0.03	—[b]	*ste13-1 dap2-1*
	B	0.05	0.05	—	*ste13-1 dap2-1*
	C	3.58	0.94	26.2	*STE13 DAP2*
	D	3.34	0.89	26.6	*STE13 DAP2*
12	A	2.48	0.03	1.2	*ste13-1 DAP2*
	B	0.95	0.83	87.4	*STE13 dap2-1*
	C	2.35	0.02	0.9	*ste13-1 DAP2*
	D	1.07	1.05	98.1	*STE13 dap2-1*

[a] Cells were grown at 30°C in YPD medium for 48 h. Crude extracts were prepared and dipeptidyl aminopeptidase activity was measured at 37°C as outlined under Materials and Methods.
[b] —, No dipeptidylaminopeptidase yscIV and yscV activities detectable.

done at 23°C; otherwise enzyme activity was measured at 37°C.

Proteinase yscA was measured after activation of its activity by the method of Saheki and Holzer (26, 27) with denatured hemoglobin as a substrate. The trichloroacetic acid-soluble product was determined by the modified Folin colorimetric method of McDonald and Chen (23). Proteinase yscB activity was determined by the method of Saheki and Holzer (26) with Hide powder azure as a substrate in extracts activated by addition of 0.2% sodium dodecyl sulfate (16). Carboxypeptidase yscY activity was assayed by the method of Aibara et al. (5) with benzoyl-Tyr-4-nitroanilide as the substrate. As a modification of the assay, 0.5% sodium deoxycholate was added (7). Carboxypeptidase yscS was determined by the method of Wolf and Weiser (40) with benzyloxycarbonyl-Gly-Leu as the substrate. Phenylmethylsulfonyl fluoride (1 mM) was added to inhibit carboxypeptidase yscY (38). Proteinase yscD activity was determined by the method of Achstetter et al. (2) with benzyloxycarbonyl-Pro-Phe-Arg-4-nitroanilide or acetyl-Ala-Ala-Pro-Ala-4-nitroanilide as the substrate.

Protein determination. The protein content of crude extracts was determined by the method of Lowry et al. (21) after boiling the samples with 0.1% sodium dodecyl sulfate for 5 min. Bovine serum albumin was used as the standard.

Production of biologically active α-factor pheromone. Production of α-factor pheromone was measured as outlined (10) with the following modifications. The assay mixture contained 4 ml of culture supernatant from *MATα* cells previously grown for 48 h on mineral liquid medium, 1 ml of YPD medium and 0.5 ml of a cell suspension (2×10^7 cells/ml) of the tester strain. As the tester strain, we used strain RC631 (*sst2*) (9), which responds not only to mature α-factor but also to incompletely processed forms of the pheromone (19).

Mutagenesis. Mutagenesis was performed with ethyl methanesulfonate as outlined by Fink (11).

Genetic techniques. The methods used for genetic analysis have been described by Hawthorne and Mortimer (15).

Isolation of dipeptidyl aminopeptidase yscV mutants. Isolation of dipeptidyl aminopeptidase yscV mutants was performed by a modification of the method used for the isolation of carboxypeptidase yscY mutants (39). Cells from a mutagenized culture of strain A2S3 or strain PSA26 were plated on YPD medium and grown at 23°C until small colonies were visible. Cells were then replica-plated onto YPD medium in glass petri dishes and allowed to grow further at 23°C. When the colonies had reached a sufficient size, cells were incubated with 10 ml of chloroform. The solvent was evaporated within about 15 min. The cells were then incubated at 37°C for 1 to 3 h to inactivate any thermosensitive enzyme generated by mutagenesis. The presence or absence of dipeptidyl aminopeptidase yscV activity in the colonies was monitored by pouring a substrate-containing staining mixture on top of the colonies. This mixture was prepared by adding 13.5 ml of 1% melted agar in 0.2 M Tris hydrochloride buffer, pH 7.0, to 1.5 ml of a solution containing 3 mg of Ala-Pro-4-methoxy-β-napththylamide and 10 mg of fast garnet GBC in dimethyl sulfoxide. The plates were incubated at room temperature until most of the colonies had acquired a deep red color (usually about 10 to 15 min). Colonies which did not stain or which stained only weakly were picked from the master plate, and single colonies were isolated and retested for their staining ability. Mutant candidates were tested biochemically for dipeptidyl aminopeptidase yscV activity.

TABLE 3. Dipeptidyl aminopeptidase yscV levels in crude extracts of segregants of the cross of strains DPSI-4A (*ste13-1 dap2-1*) × A2S3 (*ste13-1 DAP2*)[a]

Tetrad	Spore	Dipeptidyl aminopeptidase yscV activity (mU/mg)
1	A	0.07
	B	0.06
	C	2.27
	D	2.92
2	A	0.06
	B	0.02
	C	2.75
	D	2.07
4	A	2.72
	B	2.54
	C	0.08
	D	0.10
6	A	2.69
	B	0.10
	C	0.07
	D	2.21
8	A	2.08
	B	0.08
	C	0.09
	D	2.59

[a] Cells were grown at 30°C in YPD medium for 48 h. Crude extracts were prepared and dipeptidyl amino-peptidase activity was measured at 37°C as discribed in Materials and Methods.

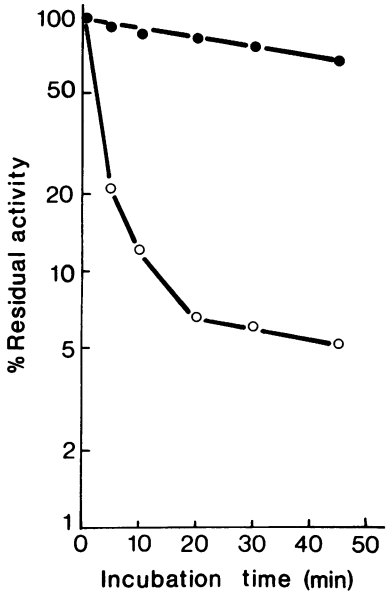

FIG. 1. Thermal inactivation of dipeptidyl aminopeptidase yscV in crude extracts from strain DPS-148 (○), bearing a thermolabile enzyme, and wild-type strain A2S3 (●). Crude extracts were prepared from cells grown in YPD medium at 23°C and used as the enzyme source. Samples of crude extracts were incubated in 0.1 M Tris hydrochloride buffer, pH 7.0, at 45°C for the time periods indicated and then immediately cooled in an ice bath. The remaining enzyme activity was assayed at 23°C.

RESULTS

Of about 18,000 plated mutagenized colonies which were already deficient in dipeptidyl aminopeptidase yscIV, we isolated 64 nonstaining and 23 weakly staining clones. These colonies were picked from the master plate, grown, and restreaked. Biochemical tests with Ala-Pro-4-nitroanilide as the substrate showed very good correlation between degree of staining and amount of dipeptidyl aminopeptidase yscV activity present in strains; nonstaining colonies were devoid of the activity, and weakly staining colonies carried 20 to 80% of wild-type activity (not shown). Mutant strains devoid of activity were named DPS-1 through DPS-64.

Strain DPS-14 was crossed with strain AP-116, which has normal dipeptidyl aminopeptidase yscIV and yscV activities. The resulting diploids showed both activities, dipeptidyl aminopeptidase yscIV and dipeptidyl aminopeptidase yscV, indicating that the mutation analyzed was recessive. Diploids were sporulated, asci were dissected, and the spore clones were germinated and analyzed for dipeptidyl aminopeptidase activity in crude extracts. In 18 tetrads analyzed, dipeptidyl aminopeptidase yscIV and dipeptidyl aminopeptidase yscV segregated independently. Table 2 shows the results corresponding to an ascus of parental ditype and to an ascus of nonparental ditype. In the first case, two spores (4A and 4B) had no dipeptidyl aminopeptidase activity, while the others (4C and 4D) had inherited both dipeptidyl aminopeptidases. After being heated for 30 min at 60°C, dipeptidyl aminopeptidase yscV was completely inactivated (7), while the activity due to dipeptidyl aminopeptidase yscIV remained almost unchanged and accounted for about 30% of the total activity (Table 2). In tetrad 12, each spore inherited either dipeptidyl aminopeptidase yscIV or dipeptidyl aminopeptidase yscV (compare specific activity before and after heating), demonstrating independent segregation of both activities. No linkage of the two genes was apparent.

To confirm that mutant strain DPS-14 carried a single chromosomal mutation which affected dipeptidyl aminopeptidase yscV, segregant DPSI-4A (*ste13 dap2*) of the cross DPS-14 × AP-116 was further crossed with strain A2S3, deficient in dipeptidyl aminopeptidase yscIV activity (*ste13*), to obtain asci in which all four spores were devoid of this enzyme. Tetrad analysis of the diploid showed a 2+:2− segregation of dipeptidyl aminopeptidase yscV activity in the 27 asci tested (Table 3). This fact confirmed that mutant strain DPS-14 indeed bore a single chromosomal mutation resulting in a deficiency in dipeptidyl aminopeptidase yscV. Other proteinases (yscA, yscB, and yscD) and carboxypeptidases yscY and yscS as well as aminopeptidase yscII were shown to segregate independently of both dipeptidyl aminopeptidase activities (not shown).

Complementation tests between the mutants revealed that they fell into one complementation group, which was called *dap2*.

As none of the nonstaining mutant strains exhibited a thermolabile dipeptidyl aminopeptidase yscV activity, we also incubated crude enzyme samples of the weakly staining mutant strains and wild-type cells at 45°C for various periods of time. The enzyme activity of strain DPS-148 was rapidly inactivated under these conditions (Fig. 1). This indicated an altered dipeptidyl aminopeptidase yscV as a result of the mutation. The behavior of dipeptidyl aminopeptidase yscV in the mutant strongly points to a mutation in the structural gene of the enzyme. Strain DPS-148, carrying the thermola-

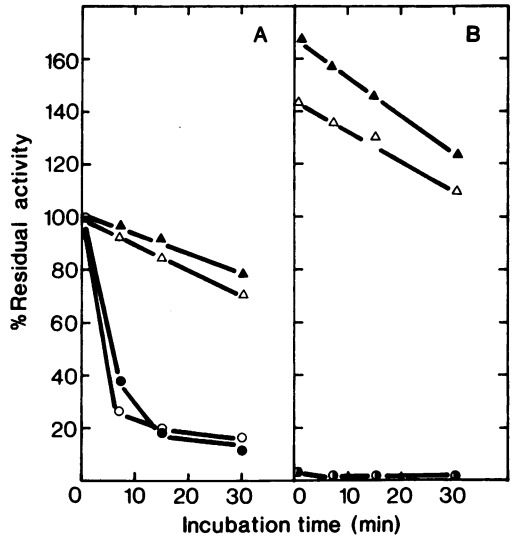

FIG. 2. Thermal inactivation of dipeptidyl aminopeptidase yscV in crude extracts (A) and crude extract fractions solubilized with octyl-β-D-glucopyranoside (B) from segregants of a tetrad derived from a diploid (DPSIV) carrying a wild-type and a mutant allele. Crude extracts and detergent-solubilized fractions were prepared from the four segregants of a tetrad derived from diploid cells of DPSIV grown in YPD medium at 23°C and used as the source of enzyme. Each sample was kept in 0.1 M Tris hydrochloride buffer, pH 7.0, at 40°C for the time periods indicated and then immediately cooled in an ice bath. The remaining enzyme activity was assayed at 23°C. Symbols: ●, strain DPSIV-4A (*ste13-1 dap2-2*); ○, strain DPSIV-4B (*ste13-1 dap2-2*); △, strain DPSIV-4C (*ste13-1 DAP2*); ▲, strain DPSIV-4D (*ste13-1 DAP2*).

bile dipeptidyl aminopeptidase yscV activity, was crossed with strain DPSI-8A, which has normal dipeptidyl aminopeptidase yscV activity but lacks dipeptidyl aminopeptidase yscIV. The diploids obtained were sporulated, and asci were dissected and germinated. All 40 ascopore clones tested for their ability to hydrolyze the substrate Ala-Pro-4-nitroanilide showed a 4+:0− segregation of activity when extracts prepared from cells grown at 23°C were tested at 23°C, whereas they showed a 2+:2− segregation when extracts were heated at 40°C for 15 min (Fig. 2A). This result indicates that strain DPS-148 carried a single chromosomal mutation.

As dipeptidyl aminopeptidase yscV is bound to the vacuolar membrane, we wanted to test the possibility that an alteration of the surrounding membrane (28) had caused the thermal instability of the mutant enzyme. Dipeptidyl aminopeptidase yscV was solubilized by octyl-β-D-glucopyranoside in the crude extracts of the above-mentioned tetrads, and the thermal stability of the enzyme was examined before and after detergent treatment (Fig. 2). More than 80% of dipeptidyl aminopeptidase yscV activity can be solubilized from wild-type cells without loss of activity under the conditions used (30). Membrane-bound dipeptidyl aminopeptidase activity showed a 2+:2− segregation of heat resistance at 40°C and thermolabile activity (Fig. 2A). After solubilization the enzyme present in the two wild-type spores (4C and 4D) was activated by the detergent and became slightly more heat sensitive than before solubilization, typical behavior for wild-type dipeptidyl aminopeptidase yscV. However, the enzyme present in the two mutant spores (4A and 4B) was rendered extremely thermolabile after solubilization. Even at 23°C it completely lost the activity (Fig. 2B), indicating that the enzyme itself and not a membrane component had been altered by the mutation. As an additional control we measured α-mannosidase activity, a vacuolar membrane enzyme marker (34), in wild-type and mutant tetrads before and after detergent treatment of crude extracts. All spores tested showed wild-type α-mannosidase activity which did not reveal any change in heat stability after solubilization (data not shown). These results strongly support the idea that no general damage of the vacuolar membrane took place, which might have rendered vacuolar membrane-bound enzymes more thermosensitive. In addition, a heterozygous diploid carrying the wild-type gene and the mutated gene showed 50% of dipeptidyl aminopeptidase yscV activity remaining after the extracts were heated, indicating that the wild-type gene product was not affected by any mutant gene product with destabilizing or inhibitory activity (Fig. 3).

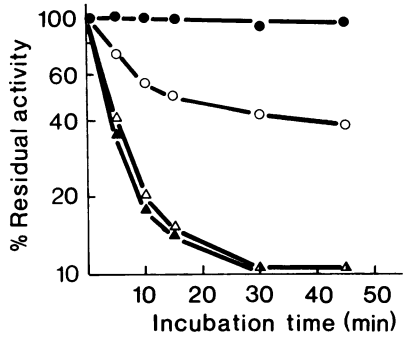

FIG. 3. Thermal inactivation of dipeptidyl aminopeptidase yscV in crude extracts of diploid strains heterozygous or homozygous for the mutant allele coding for a thermolabile dipeptidyl aminopeptidase yscV. Crude extracts were prepared from cells grown in YPD medium at 23°C and used as the enzyme source. Samples of crude extracts were incubated in 0.1 M Tris hydrochloride buffer, pH 7.0, at 40°C for the time periods indicated and then immediately cooled in ice. The remaining enzyme activity was measured at 23°C. Symbols: ●, strain DPSV (*ste13-1/ste13-1 DAP2/DAP2*); ○, strain DPSVI (*ste13-1/ste13-1 dap2-2/DAP2*); ▲, strain DPSVII (*ste13-1/ste13-1 dap2-2/dap2-2*); △, strain DPSVIII (*ste13-1/ste13-1* dap2-2/dap2-1).

TABLE 4. Gene dosage effect on dipeptidyl aminopeptidase yscV activity[a]

Strain	Genotype	Sp act (mU/mg)
DPSIX	*ste13-1 DAP2* / *ste13-1 DAP2*	2.13
DPSX	*ste13-1 DAP2* / *ste13-1 dap2-1*	1.11
DPSXI	*ste13-1 dap2-1* / *ste13-1 dap2-1*	0.08

[a] Cells were grown for 48 h at 30°C on YPD medium. Crude extracts were prepared and activity was measured at 37°C as outlined in Materials and Methods.

A segregant, DPSIV-2A, of the cross DPSI-8A × DPS-148 (bearing the thermolabile mutant allele) was crossed with a variety of mutants. Extracts of the resulting diploids grown at 23°C were tested for their ability to hydrolyze Ala-Pro-4-nitroanilide at 23°C before and after incubation of the samples at 40°C for various periods of time. Figure 3 shows an example of the complementation tests made between strains bearing the *dap2-1* and *dap2-2* mutations. The kinetics of heat inactivation in such a heterozygous diploid (DPSVIII) was indistinguishable from that of a homozygous diploid bearing the *dap2-2* mutation (DPSVII), indicating that no complementation had occurred. Similar results were obtained in all other cases (data not shown). Thus, all the mutants isolated fell into one complementation group, which defined the structural gene of the enzyme and was called *DAP2*.

The structural gene for an enzyme should show a gene dosage effect on the level of enzyme activity. Heterozygous diploids bearing a copy of the wild-type gene and a copy of the gene leading to thermolabile dipeptidyl aminopeptidase yscV showed about 50% of the activity found in a homozygous wild-type diploid after incubation at 40°C for 30 min (Fig. 3). The same gene dosage effect was observed with diploid strains carrying one of the nonthermosensitive mutant alleles (Table 4). This is consistent with the expectation that *DAP2* is the structural gene encoding dipeptidyl aminopeptidase yscV.

TABLE 5. Gene dosage effect on dipeptidyl aminopeptidase yscIV activity[a]

Strain	Genotype	Sp act (mU/mg)
DPSXII	*STE13 dap2-1* / *STE13 dap2-1*	0.77
DPSXIII	*ste13-1 dap2-1* / *STE13 dap2-1*	0.35
DPSXIV	*ste13-1 dap2-1* / *ste13-1 dap2-1*	0.04

[a] Cells were grown for 48 h at 30°C on YPD medium. Crude extracts were prepared and activity was measured at 37°C as detailed in Materials and Methods.

In previous reports (19) it has been shown that *ste13* mutants lack dipeptidyl aminopeptidase yscIV (also called dipeptidyl aminopeptidase A). The isolation of mutants devoid of dipeptidyl aminopeptidase yscV allowed us to examine the dosage effect of the chromosomal *STE13* gene on the level of dipeptidyl aminopeptidase yscIV activity in diploid strains homozygous for the absence of dipeptidyl aminopeptidase yscV. Dipeptidyl aminopeptidase yscIV activity was increased twofold by doubling the wild-type gene in a cell (Table 5). This suggests that *STE13* is the structural gene encoding dipeptidyl aminopeptidase yscIV, as was also indicated by increased levels of dipeptidyl aminopeptidase yscIV activity after introduction of a cloned *STE13* gene into cells (19).

Phenotype of dipeptidyl aminopeptidase yscV mutants. To reduce the interference of possible background markers introduced by mutagenesis in further experiments, we crossed mutant strain DPS-14 lacking dipeptidyl aminopeptidase yscV twice with a strain wild type for dipeptidyl aminopeptidase activity and a strain devoid of dipeptidyl aminopeptidase yscIV. Segregants of these crosses were used to construct diploid strains harboring different combinations of proteinase mutations (proteinase yscB [*prb1*], carboxypeptidases yscY [*prc1*] and yscS [*cps1*], and aminopeptidase yscII [*ape2*]) as well as their wild-type counterparts. These strains were used for the experiments described below.

Mutant strains lacking either dipeptidyl aminopeptidase yscV or dipeptidyl aminopeptidase yscIV as well as double mutants devoid of both enzymes grew at wild-type rates in either rich (YPD) or mineral (MV) medium at 23 or 37°C (not shown). This behavior also held true for multiple proteinase mutants lacking in addition carboxypeptidase yscY, carboxypeptidase yscS, and aminopeptidase yscII. This indicates that mitotic growth was not affected by the absence of one or the other of the two dipeptidyl aminopeptidases or by the absence of both these enzymes. Multiple-proteinase-deficient mutant strains with the genotype *ste13 dap2 prc1 cps1 ape2* were also used to test growth on the tripeptide Ala-Pro-Ala as the sole nitrogen source, a substrate of both dipeptidyl aminopeptidases in vitro. No significant difference was observed when the growth of such multiple-proteinase-deficient mutant strains was compared with that of wild-type strains carrying dipeptidyl aminopeptidase yscIV and yscV activities (not shown), suggesting that an essential nutritional role for these enzymes is either nonexistent or subtle or that there are additional enzymes that carry out redundant functions.

Drastic changes in growth conditions lead to increased intracellular protein degradation. This response is presumably designed to quickly supply the cell with amino acids for the synthesis of new proteins required for growth under the new conditions at the expense of unneeded protein. It should result in an unusually long lag phase if proteinases responsible for the degradation of proteins as a source of amino acids are missing. This was shown to be the case for some bacterial peptidase-deficient mutants (42). As dipeptidyl aminopeptidase yscV carries the rather rare specificity of splitting proline-containing peptides after the proline residue (12), we considered that the enzyme might be essential in the pathway of generating free proline from protein breakdown products. However, no differences in lag phase or growth rate were found when logarithmically growing cells lacking either dipeptidyl aminopeptidase yscV or dipeptidyl aminopeptidase yscIV as well as the double mutants lacking both enzymes were compared with wild-type strains after a shiftdown from an amino acid- and glucose-rich medium (GNA) to mineral medium devoid of amino acids (MV). Also, multiple-peptidase-deficient mutants lacking in addition to the two dipeptidyl aminopeptidases proteinase yscB, carboxypeptidase yscY, carboxypeptidase yscS, and aminopeptidase yscII did not show any alteration in the lag phase or growth rate under the above conditions (not shown).

Proteinase yscD is a nonvacuolar endopeptidase which also acts on proline-containing substrates such as benzyloxycarbonyl-Pro-Phe-Arg-4-nitroanilide, cleaving the Pro-Phe bond (2). Mutants devoid of proteinase yscD have recently been isolated and characterized (13). Strains lacking proteinase yscD in addition to the two dipeptidyl aminopeptidases also did not show any difference in growth behavior compared with the wild type.

The differentiation process of sporulation is not disturbed in diploid strains homozygous for the absence of dipeptidyl aminopeptidase yscIV, dipeptidyl aminopeptidase yscV, or both enzymes. Also, multiple-proteinase-deficient mutants homozygous for the absence of dipeptidyl aminopeptidase yscIV, dipeptidyl aminopeptidase yscV, carboxypeptidase yscY, carboxypeptidase yscS, and aminopeptidase yscII sporulate at wild-type rates (not shown). This indicates that dipeptidyl aminopeptidases yscIV and yscV do not catalyze any vital reaction during the onset of the sporulation process.

The involvement of dipeptidyl aminopeptidase yscIV in α-factor precursor processing has been unambiguously demonstrated by Julius et al. (19). *MATα ste13* mutant cells do not mate because they secrete incompletely processed forms of α-factor due to the absence of this enzyme (19). These authors also showed that the secreted, incompletely processed material was heterogeneous, the majority of molecules containing four additional amino acids and others having different numbers of additional residues, reflecting processing to different extents. Julius et al. (19) suggested that in the absence of dipeptidyl aminopeptidase yscIV, dipeptidyl aminopeptidase yscV could partly fill in for α-factor processing. Therefore, we measured production of active α-factor in *MATα* strains lacking dipeptidyl aminopeptidase yscV, dipeptidyl aminopeptidase yscIV, or both enzymes. Wild-type *MATα* strains as well as *MATα* strains devoid of dipeptidyl aminopeptidase yscV induced a prolonged arrest of growth of the *MAT***a** tester strain (more than 12 h), while *MATα* strains lacking dipeptidyl aminopeptidase yscIV were able to arrest growth of the tester strain for only 6 h, as were double mutants devoid of dipeptidyl aminopeptidase yscIV and dipeptidyl aminopeptidase yscV activities. This result indicates that dipeptidyl aminopeptidase yscV is not involved in α-factor processing in vivo. Also, the mating ability of both *MATα* and *MAT***a** strains lacking dipeptidyl aminopeptidase yscV was not affected by the absence of the enzyme, as deduced from the number of prototrophic diploids formed after crossing auxotrophic strains (not shown). In contrast to the dipeptidyl aminopeptidase yscV mutants, mating of mutants lacking dipeptidyl aminopeptidase yscIV can only be induced in the presence of externally added α-factor pheromone (8).

DISCUSSION

The results presented in this paper show that mutations in the *DAP2* gene either lead to the absence of dipeptidyl aminopeptidase yscV activity or lead to a thermolabile dipeptidyl aminopeptidase yscV activity. The sensitivity of

the enzyme to higher temperature in one of the mutants, which cannot be due to an altered membrane environment or to the appearance of a molecule inhibiting or destabilizing the enzyme, as well as the gene dosage effect on the enzyme level, strongly indicate that *DAP2* is the structural gene for dipeptidyl aminopeptidase yscV. The data presented clearly demonstrate that the X-prolyl-dipeptidyl aminopeptidase activity found previously (32) is due to two different enzymes. The outcome of the experiments shows unambiguously that the previously defined product of the *STE13* gene, which is dipeptidyl aminopeptidase yscIV (also called dipeptidyl aminopeptidase A) (19), and the product of the *DAP2* gene, which is dipeptidyl aminopeptidase yscV, are two different enzymes. Both activities segregated independently in crosses, without any apparent linkage. Neither of the enzymes depends on the presence of the other activity for expression. The gene dosage effect observed for dipeptidyl aminopeptidase yscIV activity in strains carrying combinations of mutant (*ste13*) and wild-type genes strongly supports the previous proposal (19) that *STE13* is the structural gene for dipeptidyl aminopeptidase yscIV.

Defects in dipeptidyl aminopeptidase yscV, dipeptidyl aminopeptidase yscIV, or both enzymes did not affect vegetative growth, sporulation, or adaptation to new nutritional environments of strains. The fact that *dap2* mutants did not show any obvious phenotype could be due to several reasons: (i) dipeptidyl aminopeptidase yscV might perform a highly specific proteolytic event yet to be discovered; (ii) other proteolytic enzymes present in the yeast cell can substitute for dipeptidyl aminopeptidase yscV in its absence. Extracts of *ste13 dap2* double mutants are devoid of Ala-Pro-4-nitroanilide-splitting activity, ruling out the existence of appreciable activity with overlapping specificity in vitro. However, as has been shown for dipeptidyl aminopeptidase yscIV, in vivo and in vitro specificities of proteolytic enzymes might be completely different. Whereas dipeptidyl aminopeptidase yscIV is nearly completely inactive against Glu-Ala-4-nitroanilide in vitro (P. Suárez Rendueles, T. Achstetter, and D. H. Wolf, unpublished), its intracellular function is to remove Glu-Ala and Asp-Ala residues from the α-factor pheromone precursor (19). Thus, the existence of enzymes besides dipeptidyl aminopeptidase yscV with overlapping specificity in vivo has to be considered, and only mutations in these enzymes would demonstrate the biological role of dipeptidyl aminopeptidase yscV.

The product of the *STE13* gene, dipeptidyl aminopeptidase yscIV, has been shown to be involved in the processing of the α-factor pheromone precursor (19). Two multicopy plasmids carrying different, nonhomologous DNA segments of the yeast genome were able to restore mating competence of *MATα ste13* cells, although to different extents. One plasmid carried the *STE13* structural gene, and the other carried the information for a heat-labile dipeptidyl aminopeptidase (called dipeptidyl aminopeptidase B [19]). Disruption of this gene in the chromosomal DNA (T. Stevens, unpublished) results in a mutant strain, which—as we were able to demonstrate—does not complement the *dap2* mutation and thus most likely encodes dipeptidyl aminopeptidase yscV.

A mutation in the *DAP2* gene does not affect production of active α-factor. Thus, the observation that the gene encoding vacuolar dipeptidyl aminopeptidase yscV can restore the mating competence of *MATα STE13* cells when introduced on a multicopy plasmid into cells (19) might be explained by a mislocalization of the enzyme when produced in high amounts. By this it might reach α-factor precursor and lead to illegitimate processing. Mislocalization due to overproduction has been demonstrated already for other vacuolar proteinases (25, 31).

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