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Endoproteolytic Processing of Human Parathyroid Hormone in *Saccharomyces cerevisiae* Mutants Lacking Genes Encoding the Yap3, Mkc7, and Kex2 Endoproteases

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Abstract When the human parathyroid hormone (hPTH) is expressed as a secretory product in *S. cerevisiae*, most of the secreted hPTH is internally cleaved by endoproteolytic processing. To investigate whether the yeast endoproteases such as Kex2p, Yap3p, and Mkc7p are involved in the endoproteolytic processing of hPTH in *S. cerevisiae*, hPTH was expressed in *S. cerevisiae* mutants deficient in one or two of the following well-known endoproteases such as Kex2p, Mkc7p, and Yap3p. Among these mutants, the *yap3*-disrupted (*yap3Δ*) and *yap3/mkc7*-disrupted (*yap3Δmkc7Δ*) yeasts showed a significant reduction in the extent of hPTH proteolysis. In contrast, the *mkc7*-disrupted (*mkc7Δ*) yeast did not reduce the proteolysis of hPTH as compared to the wild type. This suggests that Mkc7p is not involved in the endoproteolytic processing of hPTH. It was also found that the *kex2*-disrupted (*kex2Δ*) mutant was not able to secrete a detectable amount of hPTH.

Key words: Human parathyroid hormone, secretory production, proteolysis, endoproteases, *S. cerevisiae*

Human parathyroid hormone (hPTH), which is an 84-amino-acid polypeptide produced in the human parathyroid gland, is a principal regulator of calcium homeostasis in kidney and bones [15]. Since it is known to generate a positive Ca^{++} balance and to enhance bone formation, it has been used as an anabolic drug against post-menopausal osteoporosis [19].

The hPTH has been produced by recombinant DNA technology using a variety of host systems [6, 9, 16]. Among these, *S. cerevisiae* has many advantages over other host systems. It is non-pathogenic, produces no endotoxins, and has been cultivated on an industrial scale for centuries. In addition, a simple purification process can be adopted by secreting a foreign protein of interest extracellularly and harvesting them directly from the culture broth. However, when the hPTH is produced as a secretory product in *S. cerevisiae*, the unwanted proteolytic cleavage takes place and consequently the yield of intact hPTH is significantly reduced. Recently, therefore, we developed a simple method to solve this proteolysis problem by adding an excess amount of L-arginine to the culture medium [3]. It was also found that a *S. cerevisiae* mutant deficient in yeast aspartic protease 3 (Yap3p) reduced the extent of proteolysis of hPTH [12]. In the present study, further attempts were made to investigate whether other well-known endoproteases such as Mkc7p and Kex2p as well as Yap3p are involved in the endoproteolytic processing of hPTH in *S. cerevisiae*.

The plasmid pG10-hPTH for the expression of hPTH in *S. cerevisiae* has been described previously [3]. This plasmid consists of the *GAL10* promoter, mating factor α pre-pro leader, the gene encoding hPTH, and the *GAL7* terminator. *S. cerevisiae* L3262 (*MATa ura3-52 leu2-3,112 his4-34*) [11] was used as the parental strain mainly to construct the mutant strains deficient in endoprotease(s).

The *yap3* disruptant of L3262 was constructed as described previously [12]. The strains disrupted in the *MKC7* gene locus or in the *KEX2* gene locus were constructed by a one-step disruption technique [17]. For disruption of the *MKC7*, the 1.6 kb *MKC7* gene fragment was obtained using the polymerase chain reaction (PCR). The 0.9 kb *SalI/NdeI* DNA fragment containing most of the *MKC7* coding region was deleted from the *MKC7* gene fragment and then it was replaced by a 2.0 kb *LEU2* gene from YEp351 [7]. For the *KEX2* gene disruption, a 1.1 kb *NdeI/BglII* fragment containing an internal part of the *KEX2* coding region was deleted from the 1.8 kb *KEX2* gene fragment, which was obtained by PCR, and then it was replaced with the *LEU2* gene. Transformation of *S. cerevisiae* L3262 with a 2.8 kb DNA fragment containing the disrupted *MKC7* gene (*mkc7::LEU2*) or with a 2.6 kb

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DNA fragment containing the disrupted *KEX2* gene (*kex2::LEU2*) were carried out by the modified lithium acetate method [8]. The disruption of *MKC7* and *KEX2* genes in the resulting Leu$^+$ transformants was confirmed by Southern blot analysis. In order to construct a *mkc7/yap3* double disruptant, disruption of the *MKC7* gene in the *yap3* disruptant was carried out by using the double fusion PCR [1]. At the first fusion reaction, a 3.65 kb fusion DNA fragment was generated, in which a 250 bp N-terminal fragment of the *MKC7* gene was fused with a 3.4 kb *HIS4* gene. At the second fusion reaction, the 4.0 kb final fusion product, in which the *HIS4* gene was flanked by a 250 bp N-terminal and a 350 bp C-terminal fragments of *MKC7* gene, was generated and used to transform the *yap3* disruptant. Disruption of the *MKC7* gene in the obtained His$^+$ transformants was confirmed by Southern blot analysis. The wild-type and mutant strains used in this study are shown in Table 1.

The YPD medium (1% yeast extract, 2% bactopeptone, and 2% glucose) was used mostly for cultivating the host and yeast transformants. The YNBCAD medium (0.67% yeast nitrogen base without amino acids, 2% glucose, and 0.5% casamino acids) was used for selecting the yeast transformants and also for the seed culture. For the induction of the hPTH gene, the yeast transformants were grown in shake-flasks containing the YPDG medium (1% yeast extract, 2% bactopeptone, 1% glucose, and 1% galactose) at 30°C. Yeast extract, bactopeptone, yeast nitrogen base without amino acids, and casamino acids were purchased from Difco (Detroit, U.S.A.).

A culture supernatant obtained after centrifugation of 200 µl culture broth was treated with 20 µl 100% trichloroacetic acid to precipitate the extracellular proteins. Then, the precipitate was separated by centrifugation at 12,000 ×g for 10 min. After washing the precipitate with cold acetone, it was suspended in a 10 µl 1 × Laemmli lysis buffer and boiled for 15 min. The proteins were separated by SDS-PAGE and were stained with Coomassie brilliant blue R-250.

The amount of hPTH was measured by scanning the hPTH protein bands in SDS-PAGE using a densitometer (Bio-Rad model GS-700 Imaging Densitometer, CA, U.S.A.), using the purified hPTH (Sigma) as a standard. The cell growth was monitored by measuring absorbance at 600 nm.

When foreign proteins are expressed as secretory products in yeast, the aberrant proteolytic processing often takes place. Among the endoproteases related to the proteolytic processing in yeast, the best characterized endoprotease is the Kex2p, which was recognized for its ability to cleave the precursors of a mating pheromone and killer toxin at certain dibasic sites [10]. Also, a yeast gene encoding Yap3p (yeast aspartic protease 3) was identified as a multicopy suppressor of the pro-α-factor processing defect of a *kex2* deficient *S. cerevisae* mutant [5]. Recently, the *MKC7* gene encoding a third endoprotease, Mkc7p, with a considerable homology to Yap3p has been identified and characterized [14]. Until now, there have been several reports on the involvement of these endoproteases in the proteolytic processing of various foreign proteins and peptides in yeast [2, 4, 12, 13, 18].

When the hPTH is expressed in *S. cerevisiae*, it is cleaved after a pair of basic amino acids in positions 25 (Arg 25) and 26 (Lys 26). It is known that such a dibasic site are recognized by Kex2p, Yap3p, and Mkc7p. To investigate whether proteolysis of hPTH is mediated by these proteases, the gene encoding hPTH was expressed in *S. cerevisiae* mutants deficient in one or two of these endoproteases (Fig. 1). Among these mutants, *yap3*-disrupted (SLH11) and *yap3/mkc7*-disrupted (SLH14) mutants showed a significant reduction in the extent of hPTH proteolysis. In these cases, the cleavage occurred at a low frequency, such as ca. 80% of the secreted hPTH remained intact after 48 h of culture. On the other hand, the *mkc7*-disrupted (SLH12) yeast did not reduce the proteolysis of hPTH compared to the wild-type (ca. 90% of the secreted hPTH was cleaved), indicating that Mkc7p was not involved in the endoproteolytic processing of hPTH. It was found that the yeast mutant deficient in kex2p (SH13) secreted no detectable amount of hPTH (Fig. 1, lane 5). This was presumably due to the incomplete processing of the pro region of the mating factor α leader peptide, which prevented hPTH from being secreted out of the cells.

Figure 2 shows the cell growth of the wild-type and mutant strains. It was found that the cell growth rates of SLH11, SLH12, and SLH14 strains were similar to that of the wild-type strain, indicating that the *yap3* and *mkc7* null mutations had no effect on the cell growth rate. In contrast, the SLH13 strain was found to grow more slowly than the

Table 1. *S. cerevisiae* strains used in this study.

Strain	Disrupted genes	Genotype
L3262		*MATα ura3-52 leu2-3,112 his4-34*
SLH11	*yap3*	*MATα ura3-52 leu2-3,112 his4-34 yap3::LEU2*
SLH12	*mkc7*	*MATα ura3-52 leu2-3,112 his4-34 mkc7::LEU2*
SLH13	*kex2*	*MATα ura3-52 leu2-3,112 his4-34 kex2::LEU2*
SLH14	*yap3/mkc7*	*MATα ura3-52 leu2-3,112 his4-34 mkc7::HIS4 yap3::LEU2*

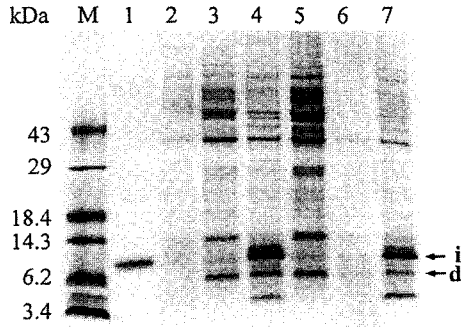

Fig. 1. SDS-PAGE analysis of extracellular proteins produced by the transformed *S. cerevisiae* L3262 wild-type (lanes 2, 3, 7) and mutants (lanes 3-6).

Lanes M, pre-stained molecular markers; 1, authentic hPTH (Sigma); 2, untransformed wild-type; 3, transformed wild-type; 4, SLH11; 5, SLH12; 6, SLH13; 7, SLH14. Bands i, intact hPTH(1-84); d, proteolytically cleaved hPTH(27-84).

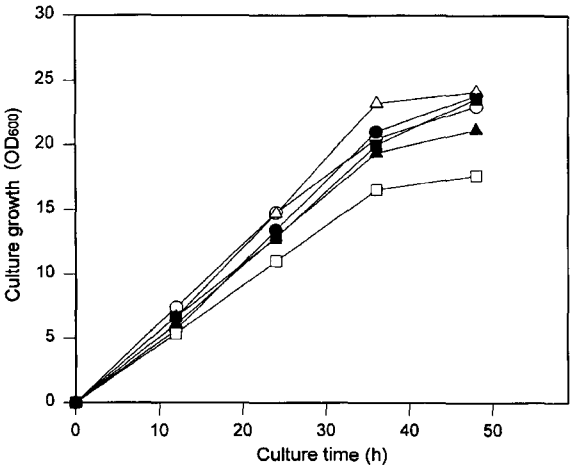

Fig. 2. Cell growth of the untransformed wild-type (○), transformed wild-type (●), SLH11 (△), SLH12 (▲), SLH13 (□), and SLH14 (■).

other strains. It is presumed that the *kex2* null mutation has a significant effect on the normal yeast physiology.

In conclusion, it was discovered that, among the yeast endoproteases Kex2p, Mkc7p, and Yap3p, Yap3p was clearly involved in the proteolysis of hPTH. However, the yeast mutant lacking the Yap3p did not suppress the proteolysis of hPTH completely, suggesting the presence of an uncharacterized endoprotease(s), involving in the endoproteolytic cleavage of hPTH in the *S. cerevisiae* secretory pathway.

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