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Enzyme activity of *O*-sialoglycoprotein endopeptidase (OSGEP) of *Saccharomyces cerevisiae* Kae1p is essential for growth, but the bacterial and mammalian OSGEP homologs can not complement the yeast *KAE1* null mutation

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***O*-sialoglycoprotein endopeptidase (OSGEP) is conserved throughout almost all species like eubacteria, archaebacteria, and eukaryotes. A homolog (*KAE1*) of *Saccharomyces cerevisiae* is the essential gene for growth of the cells. Here, we designed a complementation system of *KAE1*$^-$ lethality using an *S. cerevisiae KAE1*$^{+/-}$ heterozygous strain. After introduction of an expression plasmid pAUR123 into the heterozygote, *KAE1*$^+$ and *KAE1*$^-$ alleles were segregated by random spore analysis. The expression plasmid has a centromere sequence of *S. cerevisiae*, so is divided to each haploid cell at the same ratio. The expression of Kae1p from the plasmid could complement the lethality of *KAE1* null mutation. Alanine substitution of histidine residues in a common motif of glycoprotease M22 family abolished the complementation ability of Kae1p. Therefore, the glycoprotease activity of Kae1p is essential for growth of the yeast cells. *Escherichia coli* and mouse OSGEP homologs also have well-conserved M22 family motifs, but did not complement the *KAE1*$^-$ lethality in the yeast strain. This suggests that not only the enzymatic activity of OSGEP but also the specific interaction to other proteins is critical for function of yeast OSGEP.**

Keywords: *O*-sialoglycoprotein endopeptidase; glycoprotease M22 family; *Saccharomyces cerevisiae*; *KAE1*.

1. Introduction

O-sialoglycoprotein endopeptidase (OSGEP, EC 3.4.24.57) is the enzyme that specifically hydrolyzes the protein part of *O*-glycosylated proteins on serine or threonine residues. This enzyme has a common motif of glycoprotease M22 family, which is conserved throughout almost all species like eubacteria, archaebacteria, and eukaryotes (Fig. 1) (Reviewed in Ikeda et al., 2003). Among these homologs *Pasteurella haemolytica* A1 glycoprotease (gcp enzyme) is researched most intensively (Abdullah et al., 1991). *P. haemolytica* is a Gram-negative bacterium and is associated with bovine pneumonic pasteurellosis, a major cause of sickness and death in feedlot cattle in North America. The substrates of gcp enzyme include human cell surface glycoproteins, such as glycophorin A, CD24, CD34, CD43, CD44 and CD45, ligands for P- and L-selectins, and cranin, a brain *O*-sialoglycoprotein (Sutherland et al., 1992; Hu et al., 1994; Clark et al., 1998). *Gcp* gene of a cyanobacterium *Synehcocytis* sp. was also studied as a prokaryotic OSGEP homolog, and the mutation leads to reduced salt resistance, altered pigmentation, and cyanophycin accumulation (Zuther et al., 1998). *Gcp* gene of a Gram-positive bacterium *Staphylococcus aureus* is essential for its survival, and its sensitivity to Zn^{2+} is increased by down regulation of *gcp* expression (Zheng et al., 2005; Zheng et al., 2007). An archaeal homolog of OSGEP exhibits atypical DNA-binding properties and apurinic/apyrimidinic (AP) endonuclease activity *in vitro* (Hecker et al., 2007).

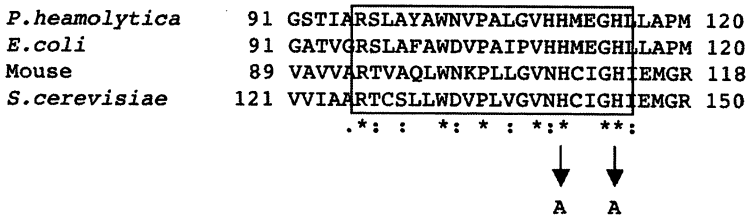

Fig. 1. Amino acid alignment of glycoprotease M22 family motifs from *P. haemolytica* A1 glycoprotease gcp, *E. coli* YgjD, mouse OSGEP and *S. cerevisiae* Kae1p. Multiple alignments were performed using CLUSTAL W Multiple Sequence Alignment Program. '*' indicates positions which have a single, fully conserved residue; ':' indicates that one of the strong groups (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY and FYW) is fully conserved; '.' indicates that one of the weaker groups (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM are HFY) is fully conserved. Potential motif for glycoprotease M22 family is boxed. Two histidine residues at amino acid number 141 and 145 of Kae1p were substituted to alanine residues (H141/145A) by site directed mutagenesis.

The OSGEP homologs are also present in mammals as well as unicellular eukaryotes. A *Saccharomyces cerevisiae* homolog (*KAE1*, kinase associated endopeptidase) is an essential gene for growth; it is known that Kae1p interacts with protein kinase Bud32p (Lopreiato et al., 2004). Pcc1p, which has homology to human cancer testis antigen, forms a complex with Kae1p and Bud32p and is related to cell cycle progression and polarized growth of the yeast (Kisseleva-Romanova et al., 2006). Furthermore, human OSGEP selectively expressed in acute promyelocytic leukemia (APL) cells, functions as a regulator of the response of APL cells to apoptosis via processing of the misfolded protein (Ng et al., 2006). The gene encoding the mammalian OSGEP is closely located to that of the major AP endonuclease and positioned in a head-to-head orientation (Seki et al., 2002; Ikeda et al., 2002). Transcription of this bidirectional gene pair is regulated by common promoter elements but physical and biochemical relationships between the two gene products are not known.

In this study we constructed a complementation system for *KAE1*$^{-}$ lethality using *S. cerevisiae* *KAE1*$^{+/-}$ heterozygous strain and expression plasmid of OSGEP homologs. Exogenous expression of Kae1p could complement the lethality of *KAE1* null mutation. M22 motif of Kae1p is essential for growth of the yeast cells. The mouse and *Escherichia coli* OSGEP homologs also have a well-conserved M22 motif, but these did not function in the yeast cells. Finally, we discussed the reasons for lack of functional complementation among OSGEP homologs from different species.

2. Materials and methods

2.1. Strains and media

S. cerevisiae KAE1 mutant (heterozygous diploid, *KAE1*$^{+/-}$) was purchased from Open biosystems (YSC1021-673728). The mutant is a derivative from parental strain BY 4743, which was generated by conjugation of haploid cells with different auxotrophy of methionine or lysine. The strain was routinely growth on YPD medium (1% yeast extract 2% polypepton, and 2% glucose) or YPAD medium (YPD supplemented with 0.04% adenine) as complete media. For selection based on auxotrophy SD medium (0.67% yeast nitrogen without amino acids and 2% glucose) containing appropriate supplements was used. All solid media contained 2% agar.

2.2. Random spore analysis

Diploid cells were spread on presporulation agar medium (0.8% yeast extract 0.3% polypepton, 10% glucose, and 2% agar) and cultured at 28°C for 2 days. Several colonies were patched on sporulation agar medium (1% CH_3COOK, 0.1% yeast extract, 0.05% glucose, and 2% agar). The culture was maintained at 28°C for 5 days. A dab of the cells was suspended in 10 μl of sterilized water, and tetrad formation was examined by microscopic observation. All cells of the lump was transferred into 300 μl of a solution containing 1.2 M sorbitol, 50 mM potassium phosphate buffer (pH 7.5), 14 mM 2-mercaptoethanol, and 0.2 mg/ml Zymolyase 100T (Seikagaku Corp.,

Tokyo, Japan), mixed gently, and incubated at 30°C for 30 min. After centrifugation the precipitate was resuspended with 300 μl of sterilized water by vigorous mixing. An aliquot (100 μl) of the spore preparation was spread on a YPD agar plate and incubated at 28°C until colonies appeared.

2.3. Construction of expression plasmid

pAUR123 (Takara Bio, Shiga, Japan) is an expression vector in *S. cerevisiae* that contains the alcohol dehydrogenase 1 gene promoter for constitutive expression in yeast and the aureobasidin A (AbA)-resistant gene as a selection marker. DNAs encoding OSGEP homologs were cloned into pAUR123 using the appropriate restriction sites. DNA of *E. coli* homolog (*ygjD*) was amplified from the genomic DNA of strain JM109 using TaKaRa Ex *Taq* polymerase (Takara Bio) and the following primers (Fw primer, 5′-acg ggt acc ATG CGT GTA CTG GGT ATT G-3′; Rv primer 5′-a ata ctc gag TTA CGC AGC CGG TAA CTC C-3′). The KAE1 gene was amplified from *S. cerevisiae* genomic DNA using the following primers: Fw primer, 5′- t gcg gtc gac AAA ATG GTC AAC TTG AAC AC -3′; Rv primer 5′- aa agg tct aga TTA ATC ACG CCA GGC TGC G -3′. Mouse Osgep was amplified from a cDNA (purchased from ResGen, Invitrogen Inc.) using the following primers: Fw primer, 5′-aat ggt acc ATG CCC GCG GTG CTG GGG TTC-3′; Rv primer 5′-ga gcc tcg agg gtG TTA GTC CCT CCA TGT C-3′. Site directional mutagenesis of the active residue of Kae1p was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, CA). The primer sequence for mutation is as follows with mutation sites underlined: 5′-CTG GTG GGA GTA AAC GCC TGC ATT GGT GCC ATC GAA ATG GGG AG-3′. Recombinant plasmids were introduced using the EZ-yeast transformation kit (Bio101 Systems, CA). Transformants were selected on YE medium containing 0.6 μg/ml of AbA.

3. Results and discussion

3.1. *KAE1* gene is essential for the growth of *S. cerevisiae*

In order to confirm whether *KAE1* is an essential gene for survival, we generated haploids from a $KAE1^{+/-}$ heterozygote. In random spore analysis two kinds of alleles (wild type and $KAE1^{-}$ allele) are segregated into haploid cells. If *KAE1* is the essential gene for survival, a strain with the $KAE1^{-}$ allele can not grow. Haploid cells derived from the $KAE1^{+/-}$ heterozygote are methionine or lysine auxotrophic. Therefore, haploid and diploid cells are discriminated by auxotrophy. The *KAE1* gene is disrupted by the *kanMX4* gene, so the $KAE1^{-}$ haploid will become G418 resistant ($G418^{R}$) if they can grow normally. On the other hand, if *KAE1* is the essential gene for survival, then the $KAE1^{-}$ haploid can not grow on every medium considered while the strain that possesses the wild type allele is sensitive to G418 ($G418^{S}$). With this experimental setup we investigated G418 resistance of 108 haploids. As a result 100 haploids were found to be sensitive to G418 and as little as 8 haploids were resistant to G418. This result shows that *KAE1* is the gene essential for survival. Less than 10% of the haploids escaped death despite the *KAE1* null mutation. The cause of this leak of lethality is not yet known, but could be interpreted after the biological function of *KAE1* is revealed.

Table 1. Random spore analysis of $KAE1^{+/-}$ heterozogote*

	Number of haploid colonies
$G418^{R}$	8 (7%)
$G418^{S}$	100 (93%)

*Experimental details are described in the text.

3.2. Complementation of $KAE1^{-}$ lethality by exogenous expression of Kae1p

We designed an experiment to complement $KAE1^{-}$ lethality by exogenous expression of OSGEP homologs in $KAE1^{+/-}$ heterozygtes and randomly analyzed spores. As a positive control we constructed a Kae1p expression plasmid using pAUR123 vector, which has an AbA-resistant gene as a selection marker. The recombinant plasmid was introduced into the $KAE1^{+/-}$ strain. Because the pAUR123 vector has a centromere sequence (CEN4) derived from *S. cerevisiae*, upon meiosis the plasmid is equally segregated into 4 spores. We performed a random spore analysis after sporulation. If the gene in the expression plasmid complements the lethality of $KAE1^{-}$, then the *KAE1*-null haploid with the plasmid is both G418- and AbA-resistant ($AbA^{R}/G418^{R}$). If the gene in the plasmid d oes not complement the $KAE1^{-}$ phenotype, then the lethality of $KAE1^{-}$ hinders

Table 2. Random spore analyses of the *KAE1*$^{+/-}$ heterozogote harboring the expression plasmid of various OSGEP homologs and the mutant*

Expression plasmid	No. of haploids tested	Haploids with *KAE1*$^{-}$ allele (AbA^{R}/ G418^{R})	Haploids with wild type allele (AbA^{R}/ G418^{S})
S. cerevisiae KAE1 (WT)	39	18 (46%)	21 (54%)
S. cerevisiae KAE1 (H141/145A)	45	0 (0%)	45 (100%)
E. coli ygjD	40	0 (0%)	40 (100%)
Mouse OSGEP	36	0 (0%)	36 (100%)

*Experimental details are described in the text.

the growth of haploid cells. Moreover, the wild type haploid harboring the expression plasmid is AbA-resistant and G418-sensitive (AbA^{R}/G418^{S}). With random spore analysis we initially spread the spores on medium containing AbA and selected out haploids that contained the plasmid. Then we confirmed that these colonies were truly haploids by auxotrophy. Furthermore, we investigated if these haploids could grow on medium containing G418. As shown in Table 2 we obtained equal yields of AbA^{R}/G418^{R} and AbA^{R}/G418^{S} colonies. This result shows that the plasmid is segregated to *KAE1*$^{-}$ haploids and wild type haploids in roughly a 1:1 ratio and that Kae1p expressed from the plasmid could complement the lethality of the *KAE1* null mutation.

3.3. M22 motif of Kae1p is essential for growth of the yeast cells

OSGEP homologs contain an evolutionally well-conserved M22 family motif and there exist histidine residues that chelate the metal ion (Fig. 1) explaining the experimental data that EDTA and 1,10-phenanthroline inhibit the gcp enzyme of *P. haemolytica* (Abdullah et al., 1992). Here we investigated whether the M22 motif in Kae1p is necessary for the growth of *S. cerevisiae* cells. We substituted two histidine residues at amino acid number 141 and 145 in the M22 motif of Kae1p by alanine (H141/145A) by site directed mutagenesis, and introduced the mutated plasmid into the *KAE1*$^{+/-}$ strain. By random spore analysis we could not obtain a *KAE1*$^{-}$ haploid with the mutant plasmid (AbA^{R}/G418^{R}) (Table 2). This indicates that M22 motif-deficient Kae1p could not complement *KAE1*$^{-}$ lethality. Therefore, the *O*-sialoglycoprotein endopeptidase activity of Kae1p is essential for growth of yeast cells.

3.4. Complementation ability of OSGEP homologs to *KAE1* null mutation

Amino acid sequences of OSGEP homologs are conserved in a broad range of organisms (Ikeda et al., 2003). In order to study whether the functions of these homologs are also conserved, we used a *KAE1*$^{+/-}$ yeast strain in a complementation experiment. DNA of *E. coli* (ygjD) and mouse OSGEP homologs were constructed into expression vector pAUR123 and each construct was introduced into the *KAE1*$^{+/-}$ strain. By random spore analysis we could not obtain *KAE1*$^{-}$ haploids with the expression plasmid (Table 2). Therefore it was concluded that *E. coli* and mouse OSGEP homologs could not complement the lethality of *KAE1* null mutation in the yeast strain. Due to the lack of functional complementation we speculate that the substrate specificity of the OSGEP enzyme differs among species. Furthermore, mammalian species possess another type of OSGEP homolog (OSGEPL1) that have a conserved motif of the peptidase M22 family protein (Ikeda et al., 2003). Also, during the evolution of OSGEP homologs from a common ancestor, each gene might have obtained a function other than glycoprotease. In fact, Kae1p of budding yeast interact with many proteins and function as a transcription factor (Lopreiato et al., 2004; Kisseleva-Romanova et al., 2006). This suggests that not only the enzymatic activity but also

the specific protein-protein interaction is necessary for the critical role of the yeast OSGEP. The *KAE1*⁻ complementation system constructed in this study would provide a useful tool for analyzing functional domains of the yeast OSGEP.

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