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Shared functions *in vivo* of a glycosyl-phosphatidylinositol-linked aspartyl protease, Mkc7, and the proprotein processing protease Kex2 in yeast

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ABSTRACT **The *MKC7* gene was isolated as a multicopy suppressor of the cold-sensitive growth phenotype of a yeast *kex2* mutant, which lacks the protease that cleaves pro-α-factor and other secretory proproteins at pairs of basic residues in a late Golgi compartment in yeast. *MKC7* encodes an aspartyl protease most closely related to product of the *YAP3* gene, a previously isolated multicopy suppressor of the pro-α-factor processing defect of a *kex2* null. Multicopy *MKC7* suppressed the α-specific mating defect of a *kex2* null as well as multicopy *YAP3* did, but multicopy *YAP3* was a relatively weak suppressor of *kex2* cold sensitivity. Overexpression of *MKC7* resulted in production of a membrane-associated proteolytic activity that cleaved an internally quenched fluorogenic peptide substrate on the carboxyl side of a Lys-Arg site. Treatment with phosphatidylinositol-specific phospholipase C shifted Mkc7 activity from the detergent to the aqueous phase in a Triton X-114 phase separation, indicating that membrane attachment of Mkc7 is mediated by a glycosyl-phosphatidylinositol anchor. Although disruption of *MKC7* or *YAP3* alone resulted in no observable phenotype, *mkc7 yap3* double disruptants exhibited impaired growth at 37°C. Disruption of *MKC7* and *YAP3* in a *kex2* null mutant resulted in profound temperature sensitivity and more generalized cold sensitivity. The synergism of *mkc7, yap3,* and *kex2* null mutations argues that Mkc7 and Yap3 are authentic processing enzymes whose functions overlap those of Kex2 *in vivo*.**

The *Saccharomyces cerevisiae* Kex2 protease is a Ca^{2+}-dependent transmembrane serine protease that cleaves secretory proproteins on the carboxyl side of Lys-Arg and Arg-Arg in a late Golgi compartment (1, 2). Kex2 is the prototype of a family of eukaryotic proteases implicated in processing precursors of peptide hormones, neuropeptides, viral envelope glycoproteins, growth factors, and growth factor receptors at basic-residue-containing sites (3). Several reports have suggested that aspartyl proteases may also be involved in proprotein processing in neuroendocrine cells (4, 5). A yeast gene, *YAP3*, that encodes an aspartyl protease was identified as a multicopy suppressor of the pro-α-factor processing defect of a *kex2* mutant strain (6) and of *sex1* mutations that blocked processing of a heterologous substrate, anglerfish prosomatostatin, at a single Arg-type cleavage site when expressed in yeast (7). However, because *yap3* null mutations have no effect on normal yeast physiology, it has been unclear what the role of the *YAP3* product (Yap3) is in yeast and even whether it is a processing or degradative enzyme. In addition to α-specific sterility, *kex2* mutants exhibit additional phenotypes, including cold-sensitive growth (Cs^- phenotype; R.S.F. and J. Thorner, unpublished data; ref. 8). We report the identification and characterization of the *MKC7* gene,‡ encoding an aspartyl protease most closely related in sequence to Yap3, as a multicopy suppressor of the *kex2* Cs^- phenotype. Combining disruptions of the *MKC7, YAP3*, and *KEX2* genes resulted in clear growth phenotypes at low and high temperatures. The results suggest that the Mkc7 and Yap3 proteases are, like Kex2, proprotein processing enzymes that have important roles in normal yeast physiology.

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MATERIALS AND METHODS

Yeast Media and Strains. Liquid and solid rich media supplemented with 1% adenine sulfate (YPAD) and synthetic complete medium (SDC) were as described (9). *kex2* null mutations were as described (10). *kex2* null strains were not cold-sensitive on SDC but were on modified SDC, in which (*i*) proline (1%) replaced $(NH_4)_2SO_4$ as a nitrogen source, (*ii*) KH_2PO_4 was 1.0 mM, (*iii*) $MgSO_4$ was 0.5 mM, and (*iv*) pH was buffered at 6.5 with 50 mM Mes (Calbiochem).

All yeast strains were derived from the W303 background (R. Rothstein, Columbia University; ref. 11). Strains: CRY2, *MATα, can1-100, ade2-101, his3-11,-15, leu2-3,-112, trp1-1, ura3-1*; BFY106-4D, CRY2 *kex2Δ2::HIS3*; KRY18, a *MAT***a**/*MATα* diploid heterozygous at *KEX2* (*kex2Δ2::TRP1*/+) but homozygous for all other markers in CRY2; HKY18, KRY18 *yap3Δ::LEU2*/+, *mkc7Δ::HIS3*/+; HKY19, CRY2 *kex2Δ2::TRP1*; HKY20, CRY2 *yap3Δ::LEU2*; HKY21, CRY2 *mkc7Δ::HIS3*; HKY22, CRY2 *kex2Δ2::TRP1, yap3Δ::LEU2*; HKY23, CRY2 *kex2Δ2::TRP1, mkc7Δ::HIS3*; HKY24, CRY2 *yap3Δ::LEU2, mkc7Δ::HIS3*; HKY25, CRY2 *kex2Δ2::TRP1, mkc7Δ::HIS3, yap3Δ::LEU2*; HKY26, CRY2 *yap3Δ::LEU2, mkc7Δ::HIS3, pep4::HIS3, prb1Δ::LEU2*; HKY27, CRY2 *MAT***a**, *kex2Δ2::TRP1, yap3Δ::LEU2, mkc7Δ::HIS3*; KRY36-5B, CRY2 *pep4::HIS3, prb1Δ::LEU2*. Strains HKY19 to HKY25 were obtained by sporulation of HKY18. HKY26 was obtained from a cross of HKY27 with KRY36-5B.

Plasmids, Cloning, and DNA Sequencing. DNA manipulations followed standard methods (12). A library of yeast genomic *Sau*3A partial fragments inserted into the *Bam*HI site of episomal *LEU2* vector YEp13 was as described (13). Genomic inserts were subcloned into plasmid pHK306, a multicopy yeast vector constructed by ligating the 3.3-kb *Sca* I fragment of yeast vector YEp352 (14), containing the 2-μm replication origin and the *URA3* gene to the 3.2-kb *Sca* I fragment of pRS306 (15). pHKMKC7 is a 4.3-kb *Spe* I–*Sma* I fragment containing the *MKC7* gene inserted into plasmid pHK306. pHKYAP3 is a *Sal* I–*Xba* I fragment containing the *YAP3* gene inserted into plasmid pHK306. Nucleotide sequence was determined by using double-stranded exonuclease III deletion clones as templates by the automated dideoxynucle-

Abbreviations: PI-PLC, phosphatidylinositol-specific phospholipase C; GPI, glycosyl-phosphatidylinositol.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. U14733).

otide chain-termination method. For overexpression of *MKC7*, a fragment containing the *MKC7* structural gene was placed under the control of the *TDH3* promoter by insertion into multicopy vector pG5 (16), creating plasmid pGMKC7. This construction will be described in detail elsewhere (unpublished data).

Null Mutations of *YAP3* and *MKC7*. Sequential one-step gene disruptions (17) of *YAP3* and *MKC7* in strain KRY18 to create strain HKY18 were confirmed by hybridization analysis of restriction digests of genomic DNA. *YAP3* was disrupted by replacement with *LEU2* gene as described (6). *MKC7* was disrupted by replacing an internal 1.1-kb *Nhe* I–*Nde* I segment (61% of the *MKC7* coding sequence) with a 1.8-kb *Bam*HI fragment containing the *HIS3* gene.

Quantitative Mating Assays. Mating assays were performed essentially as described (2). The mating efficiency of the *MATα* strain was calculated as the number of diploids formed, divided by the number of diploids plus remaining *MATα* cells.

Assay of Mkc7 Proteolytic Activity. Reaction mixtures (20 μl) were 100 mM sodium citrate, pH 4.0/5 mM $CaCl_2$/0.01% Triton X-100/20 μM internally quenched fluorogenic peptide substrate (18) Arg-GluEDANS-Ser-Leu-Asp-Lys-Arg-Glu-Ala-Glu-Ala-LysDABCYL-Arg, where EDANS is (5-aminoethylamino)naphthalene-1-sulfonic acid and DABCYL is (4-dimethylaminophenyl)azobenzene carboxylic acid. After enzyme addition, reaction mixtures were incubated at 37°C for 30 min and terminated by addition of 680 μl of 1 M Tris (pH 10). Fluorescence was determined with a Perkin–Elmer LS-5B fluorimeter (λ_{ex}, 338 nm; λ_{em}, 490 nm). One unit of activity was defined as an increase in fluorescence equivalent to 1 pmol of GluEDANS per min.

Phosphatidylinositol-Specific Phospholipase C (PI-PLC) Digestion. Mkc7 was purified from Triton X-100-solubilized membrane proteins by the following chromatographic steps: Q-Sepharose, Cu^{2+}-charged chelating Sepharose, and Sephacryl S-300 (resins from Pharmacia; the details of the purification and characterization will be described elsewhere; unpublished data). For the phase separation, bound Mkc7 was washed and eluted from a second Cu^{2+}-charged chelating Sepharose column by using buffer containing Triton X-114 (0.1%). Purified Mkc7 (1000 units) was incubated with 0.05 unit of *Bacillus cereus* PI-PLC (Boehringer Mannheim) at 30°C for 15 min in 0.2 M NaCl/50 mM Hepes, pH 7.5/0.1% Triton X-114. After the incubation, phases were separated, the aqueous and detergent phases were extracted once more as described (19), and the proteolytic activity in each phase was measured.

RESULTS AND DISCUSSION

***kex2*Δ Cold Sensitivity.** Haploid and homozygous diploid *kex2*Δ cells underwent reversible growth arrest at or below 20°C on solid or in liquid YPAD medium (Fig. 1*A*, data not shown). At 30°C in YPAD liquid, the doubling time of *kex2*Δ cells was indistinguishable from that of *KEX2*$^+$ cells. *kex2*Δ cells grown at 30°C in YPAD ceased dividing within three doublings after being shifted to 16°C (data not shown). Cold sensitivity was not observed on synthetic medium, and addition of ammonium sulfate or other salts present in SDC to YPAD or adjusting the pH to <4 suppressed the cold-sensitive phenotype (data not shown).

Growth arrest was accompanied by the appearance of aberrant morphology (Fig. 1*B*). Grown at 30°C, *kex2*Δ cells were rounder than *KEX2*$^+$ cells but similar in size. After shifting *kex2*Δ strains to 16°C, mother cells became considerably larger (up to 20-fold larger in volume), with one or more buds or attached daughter cells (≈60% singly budded; ≈30%, multiply budded), and exhibited chitin and actin delocalization (Fig. 1*B*, parts b and f). Total actin staining was increased and abundant actin cortical patches appeared in budded mother cells instead of being exclusively localized to buds, as in wild-type cells (ref. 20; Fig. 1*B*, parts a–c). Calcofluor staining of *kex2* mutant cells revealed extensive chitin deposition not only in bud scars but throughout the surface of both mother cells and buds. In contrast, the bulk of chitin synthesis is normally limited to bud scars in wild-type cells (ref. 21; Fig. 1*B*, parts e–g). These morphological features of *kex2*Δ cells arrested at low temperature are suggestive of a defect in polarized growth (22).

Multicopy Suppression of *kex2*Δ Cold Sensitivity. The role of Kex2 as a proprotein processing enzyme along with the defects observed in *kex2*Δ null mutants at 16°C suggested that normal yeast growth at low temperatures requires the function of processed forms of one or more unidentified Kex2 substrates. A multicopy suppression approach (23) was taken to identify genes encoding substrates whose overexpression might overcome a defect in processing, although it was expected that other kinds of multicopy suppressors might also be isolated.

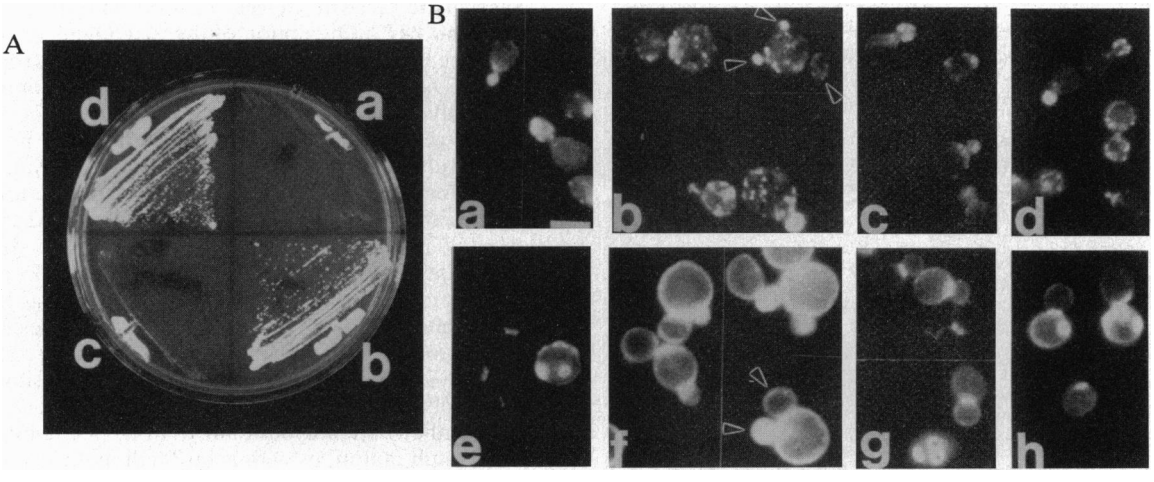


FIG. 1. Suppression of growth and morphological phenotypes of a *kex2* null mutant at 16°C by multicopy *MKC7*. (*A*) Strain BFY106-4D (*kex2*Δ), untransformed or transformed with a multicopy plasmid, and control strain CRY2 (*KEX2*$^+$) were grown on YPAD plates at 16°C for 5 days. Sectors: a, BFY106-4D without a plasmid; b, CRY2; c, BFY106-4D containing vector pHK306; d, BFY106-4D containing *MKC7*-expressing plasmid pHKMKC7. (*B*) Strains CRY2 (parts a and e), BFY106-4D (parts b and f), and BFY106-4D containing pHKMKC7 (parts d and h) were grown for 80 h after shifting from 30°C to 16°C. In parts c and g, BFY106-4D in logarithmic-phase growth at 30°C in YPAD liquid medium was a control. Cells were fixed with 4% (wt/vol) formaldehyde and stained as described (9). In parts a–d, actin was stained with rhodamine phalloidin (Molecular Probes) at 0.5 μg/ml. In parts e–h, chitin was stained with Calcofluor White (Sigma) at 0.1 μg/ml. Note that haploid strains of the W303 background (part e) exhibit random budding. Arrowheads indicate multiple buds. (Bar = 5 μm.)

Modified synthetic medium lacking leucine was used to select Cs^+ colonies after transformation of strain BFY106-4D with a multicopy yeast genomic library. Transformants secreting wild-type levels of α-factor were discarded as likely containing the wild-type *KEX2* gene. Seven of ≈10,000 total Leu^+ transformants exhibited a plasmid-dependent Cs^+ phenotype and a lack of α-factor secretion. Restriction analysis of the recovered plasmids revealed three chromosomal segments, corresponding to three genes designated *MKC1, MKC4*, and *MKC7* (for multicopy suppressor of *kex2* cold sensitivity). *MKC7* was the strongest multicopy suppressor. *MKC1* and *MKC4* encoded potential secretory proteins that may represent substrate molecules (data not shown) and will not be discussed further. Multicopy *MKC7* restored a nearly wild-type growth rate (Fig. 1*A* and Table 1) and morphology (Fig. 1*B*, parts d and h) to *kex2*Δ cells at 16°C, suggesting that the defects in growth and morphology observed in *kex2* mutants at 16°C are linked.

MKC7* Encodes an Aspartyl Protease Structurally and Functionally Similar to Yap3.** The predicted 596-aa product of the *MKC7* open reading frame exhibited significant sequence similarity to diverse aspartyl proteases (Fig. 2 *B* and *C*). Segments containing the two catalytic aspartyl residues were conserved between the predicted *MKC7* product Mkc7 and all other aspartyl proteases examined (Fig. 2*C*). The yeast *YAP3* gene product Yap3 (6) exhibited the highest degree of relatedness to Mkc7 (53% identity and 73% similarity; see Fig. 2*B*). The yeast *BAR1* gene product, an aspartyl protease secreted by *MATa** haploids that degrades α-factor, was the next most closely related molecule (32% identity) (27).

Like Yap3, Mkc7 has features of a secretory protein. Mkc7 has a potential N-terminal signal peptide with a predicted cleavage site after Ala-22 (35) and nine potential Asn glycosylation sites (36). Similarity between Mkc7 and Yap3 (Fig. 2*B*) extends through a C-terminal hydrophobic segment proposed to constitute a signal for addition of a glycosylphosphatidylinositol (GPI) membrane anchor to Yap3 (26).

Because the *YAP3* gene was originally identified as a multicopy suppressor of the pro-α-factor processing defect of *kex2* mutants (6), we compared the abilities of multicopy *MKC7* and *YAP3* to suppress both the α-specific sterility and the Cs^- phenotype caused by a *kex2* null mutation. Multicopy *MKC7* and multicopy *YAP3* restored the mating competence of *MATα kex2*Δ strain to the same level, ≈3000-fold above background or ≈10% of wild-type mating (Table 1). Multicopy *YAP3* also suppressed *kex2* cold sensitivity, though less well than did multicopy *MKC7* (Table 1). The most straightforward interpretation of these results is that Mkc7 and Yap3 can substitute at least partially for Kex2 in cleaving Kex2 substrates *in vivo*.

Mkc7 Is a Membrane-Associated Protease. Purified Yap3 was shown to have proteolytic activity that cleaved at clusters of basic amino acid residues (37). The *MKC7* structural gene was placed under the control of the powerful constitutive *TDH3* promoter on a multicopy plasmid. Yeast transformed with this expression plasmid produced high levels of a cell-associated proteolytic activity that cleaved a peptide substrate corresponding to the first Kex2 cleavage site in pro-α-factor (ref. 38; arrow indicates site of Kex2 cleavage), Ser-Leu-Asp-Lys-Arg-↓-Glu-Ala-Glu-Ala (Table 2). Mass spectral analysis confirmed that the Mkc7 activity cleaved on the carboxyl side of the Arg residue (data not shown). Cleavage of the substrate was undetectable in fractions from cells containing the expression vector lacking the *MKC7* structural gene. About 70% of the activity in the low-speed supernatant fraction sedimented at 100,000 × *g*, and 80% of this pelleted activity was solubilized by Triton X-100 (Table 2), behavior typical of membrane-associated proteins. The protein responsible for this activity, purified to homogeneity from detergent-solubilized membranes, corresponded to the *MKC7* gene product as determined by amino acid sequence analysis (H.K. and R.S.F., unpublished data).

Table 1. Suppression of *kex2* α-specific sterility and cold sensitivity by multicopy *MKC7* and *YAP3*

KEX2 allele	Multicopy gene	Mating efficiency, % of wild-type	Doubling time at 16°C, h
*kex2*Δ	*MKC7*	14.4 ± 2.4	8
*kex2*Δ	*YAP3*	8.6 ± 1.8	12
*kex2*Δ	None	$(3.6 \pm 1.5) \times 10^{-3}$	Arrested
KEX2	None	100	7

For mating efficiency, results of quantitative matings represent averages ±1 SD from the mean of duplicate assays. The mating efficiencies of strains containing multicopy *MKC7* and *YAP3* were determined by using two transformants. The mating efficiency of the *MATα KEX2* wild-type strain CRY2 was 91 ± 1%. For doubling time, cells were diluted to 7×10^5 cells per ml every 20 h up to 100 h after the shift to 16°C. The doubling time was measured 80 h after temperature shift.

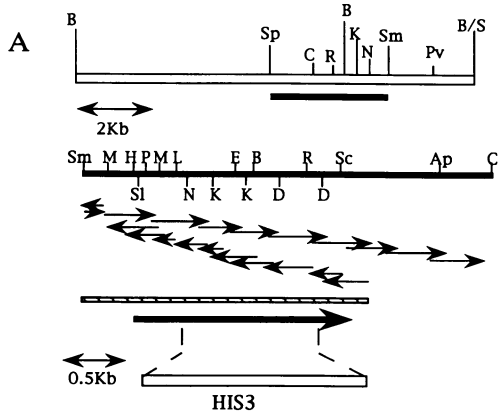
Membrane Association of Mkc7 Is Mediated by a GPI Anchor. The possibility that membrane association of Mkc7 depends on modification of the C terminus by addition of a GPI anchor was tested by assessing the effect of digestion by PI-PLC on membrane association of purified Mkc7. In a Triton X-114 phase separation, purified Mkc7 behaved like an integral membrane protein, with ≈80% of the enzyme partitioning to the detergent phase. Treatment with PI-PLC shifted the majority of the enzyme to the aqueous phase (Fig. 3), indicating that Mkc7 is anchored to the membrane through a GPI anchor (39, 40). Similarly, Yap3 has also been shown to be released from the yeast cell surface by digestion with PI-PLC (Y. Bourbonnais and D. Y. Thomas, personal communication).

The signal for GPI anchor addition includes a 15- to 20-residue hydrophobic sequence at the extreme C terminus that is removed upon addition of the anchor (25). That GPI anchor addition may be important for Mkc7 function was indicated by the fact that deletion of sequences encoding the C-terminal 16 residues of Mkc7 weakened multicopy suppression of *kex2* null cold sensitivity. Moreover, deletion of the last 107 C-terminal residues of Mkc7 eliminated suppression entirely, even though the aspartyl protease domain was still intact (data not shown). These results suggest that proper localization of the enzyme, most likely to the plasma membrane (25), is essential for its function.

Synergistic Growth Defects of Null Mutations in *KEX2, MKC7*, and *YAP3*. Disruption of the *YAP3* gene or the *MKC7* gene alone had no effect on growth of yeast cells from 16°C to 39°C (ref. 6, Fig. 4*A*, and data not shown). In contrast, *yap3 mkc7* double disruptants exhibited slow growth at 37°C (Fig. 4*A*), and at 39°C failed to form colonies (data not shown). These results demonstrate that the Yap3 and Mkc7 are redundant for a function required for high-temperature growth.

Combination of a *kex2* disruption with double disruption of both *mkc7* and *yap3* resulted in a profound growth defect even at 37°C on rich medium (Fig. 4*A*). Synergistic effects of mutations in the three genes were also observed at 16°C. Like *kex2* null mutants, *kex2 mkc7* and *kex2 yap3* double disruptants exhibited cold-sensitive growth only on YPAD. In contrast, the *mkc7 yap3 kex2* triple mutant exhibited cold-sensitive growth on both YPAD and synthetic medium (Fig. 4*B*).

The synthetic phenotypes that result from combining *mkc7* and *yap3* null mutations with a *kex2* null mutation argue that the two aspartyl proteases are in fact processing enzymes whose activity is important for normal yeast cell growth. One possibility is that Mkc7, Yap3, and Kex2 cleave a common substrate or pool of substrates whose mature form(s) is(are) required for both low- and high-temperature growth. Alternatively, Mkc7 and Yap3 on the one hand and Kex2 on the other may cleave different substrates whose mature forms are functionally redundant. The latter possibility is consistent with



B

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Mkc7P   MKLSVLTFVVDALLVCSSIVDAGV--TDFPSLPSNEVYVKMNFQKKYGSS   48
Yap3p   MKLKTVRSAVLSSLFASQVLGKIIPAANKRDDDSNSKFVKLPFHKLYGDS   50

Mkc7P   FENALDDTKGRTRLMTRDDDYELVELTNQNSFYSVELDIGTPPQKVTVLV   98
Yap3p   LENVGSDKKPEVRLLKRADGYEEIIITNQQSFYSVDLEVGTPPQNVTVLV  100
                                ↓
Mkc7P   DTGSSDLWVTGSDNPYCSTKKKDTTGSSF--KQVNKDALASVVESV--F-  143
Yap3p   DTGSSDLWIMGSDNPYCSSNSMGSSRRRVIDKRDDSSSGGSLINDINPFG  150

Mkc7P   ------TEISYDTTIVTSEATATFDSTASTSQLIDCATYGTFNTSKSSTF  187
Yap3p   WLTGTGSAIGPTATGLGGGSGTATQSVPASEATMDCQQYGTFSTSGSSTF  200

Mkc7P   NSNNTEFSIAYGDTTFASGTWGHDQLSLNDLNITGLSFAVANETNSTVGV  237
Yap3p   RSNNTYFSISYGDGTFASGTFGTDVLDLSDLNVTGLSFAVANETNSTMGV  250

Mkc7P   LGIGLPGLESTYSGVSLSSVQKSYTYNNFPMVLKNSGVIKSTAYSLFAND  287
Yap3p   LGIGLPELEVTYSGSTASHSGKAYKYDNFPIVLKNSGAIKSNTYSLYLND  300

Mkc7P   SDSKHGTILFGAVDHGKYAGDLYTIPIINTLQHRGYKDPIQFQVTLQGLG  337
Yap3p   SDAMHGTILFGAVDHSKYTGTLYTIPIVNTLSASGFSSPIQFDVTINGIG  350
                                ↓
Mkc7P   TSKGDKEDNLTTLTTTKIPVLLDSGTTISYMPTELVKMLADQVGATYSSA  387
Yap3p   ISDSGSSNK--TLTTTKIPALSDSGTTLTYLPQTVVSMIATELGAQYSSR  398

Mkc7P   YGYYIMDCIKEMEEESSIIFDFGGFYLSNWLSDFQLVTDSRSNICILGIA  437
Yap3p   IGYYVLDCPS--DDSMEIVFDFGGFHINAPLSSFILSTGT---TCLLGII  443

Mkc7P   PQSDPT-IILGDNFLANTYVVYDLDNMEISMAQANFSDDGEYIEIIESAV  486
Yap3p   PTSDDTGTILGDSFLTNAYVVYDLENLEISMAQARYNTTSENIEIITSSV  493

Mkc7P   PSALKAPGYSSTWSTYESIVSGGNMFSTAANSSISYFASTSHSATSSSSS  536
Yap3p   PSAVKAPGYTNTWSTSASIVTGGNIFTVNSSQTASF--------------  529

Mkc7P   KGQKTQTSTAALSISKSTSSTSSTGMLSPTSSSSPRKENGGHNLNPPFFA  586
Yap3p   --------SGNLTTSTASATSTSS-----------KRNVGDHIVPSLPL  559

Mkc7P   RFITAIFHHI   596
Yap3p   TLISLLFAFI   569
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C

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Sc MKC7p      81 YSVELDIGTPPQKVTVLVDTGSSDLWVTGS
Sc YAP3p      83 YSVDLEVGTPPQNVTVLVDTGSSDLWIMGS
Sc BAR1p      45 YATTLDIGTPSQSLTVLFDTGSADFWVMDS
Ca C-pepsin   69 YFTEIEIGTPGQPFKVILDTGSSNLWV---
Rm M-pepsin   42 YAIPVSIGTPGQDFLLLFDTGSSDTWV---
Sc Pep4p      36 YYTDITLGTPPQNFKVILDTGSSNLWV---
Hs Peps A     29 YFGTIGIGTPAQDFTVVFDTGSSNLWV---
Hs Cath D     33 YYGEIGIGTPPQCFTVVFDTGSSNLWV---
Hs Cath E     42 YFGTISIGSPPQNFTVIFDTGSSNLWV---
Hs Renin      43 YYGEIGIGTPPQTFKVVFDTGSSNVWV---
                   *  . .*.* *   .. ****.. *.

Sc MKC7p     352 TTKIPVLLDSGTTISYMPTELVKMLADQVG
Sc YAP3p     362 TTKIPALSDSGTTLTYLPQTVVSMIATELG
Sc BAR1p     279 TTKYPVLLDSGTSLLNAPKVIADKMASFVN
Ca C-pepsin  265 HKTGAA-IDTGTSLITLPSSLAEII-NAKI
Rm M-pepsin  251 SRPQAFTIDTGTNFFIMPSSAASKIVKAAL
Sc Pep4p     272 ESHGAA-IDTGTSLITLPSGLAEMI-NAEI
Hs Peps A    223 EGCQAI-VDTGTSLLTGPTSPIANI-QSDI
Hs Cath D    242 EGCEAI-VDTGTSLMVGPVDEVREL-QKAI
Hs Cath E    238 EGCQAI-VDTGTSLITGPSDKIKQL-QNAI
Hs Renin     242 DGCLAL-VDTGASYISGSTSSIEKL-MEAL
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FIG. 2. Structure of the *MKC7* gene and Mkc7. (*A*) In the upper restriction map of the original insert containing the *MKC7* gene, the bold bar represents the *Spe* I–*Sma* I fragment that confers functional suppression. The lower map represents the sequenced *Spe* I–*Sma* I fragment, with the orientation and extent of sequencing tracts shown by small arrows. The cross-hatched box illustrates the minimal suppressing region determined by deletion analysis. The bold arrow indicates the *MKC7* coding region (arrowhead at the C terminus), and dashed lines indicate the region replaced by the yeast *HIS3* gene. Ap, *Apa* I; B, *Bam*HI; C, *Cla* I; D, *Nde* I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa* I; K, *Kpn* I; M, *Mlu* I; N, *Nhe* I; P, *Pst* I; Pv, *Pvu* II; R, *Eco*RV; S, *Sau*3A; Sa, *Sac* I; Sc, *Sca* I; Sl, *Sal* I; Sm, *Sma* I; Sp, *Sph* I; St, *Sty* I; X, *Xba* I. (*B*) The deduced amino acid sequence of Mkc7 was aligned with that of Yap3 by the CLUSTAL V program with a PAM250 substitution matrix (24). Asterisks, amino acid identities; dots, conservative substitutions; arrows, two potential catalytic aspartyl residues; shaded boxes, elements of potential C-terminal signals for addition of GPI anchors to Mkc7 and Yap3 (25, 26). (*C*) Segments of Mkc7 were aligned (by CLUSTAL V) with segments containing the catalytic Asp residues of several other aspartyl proteases. *Sc* (*Saccharomyces cerevisiae*) Mkc7, Mkc7 residues 25–596; *Sc* Yap3, Yap3 residues 22–569 (6); *Sc* Bar1, Bar1 residues 25–587 (27); *Ca* (*Candida albicans*) C-pepsin, candida pepsin 1 precursor residues 39–380 (28); *Rm* (*Rhizomucor miehei*) M-pepsin, mucoropepsin precursor residues 23–430 (29); *Sc* Pep4, Pep4 residues 22–405 (30); *Hs* (*Homo sapiens*) Peps A, pepsinogen A residues 16–388 (31); *Hs* Cath D, cathepsin D precursor residues 19–412 (32); *Hs* Cath E, cathepsin E precursor residues 18–396 (33); *Hs* Renin, renin precursor residues 24–406 (34). D(T/S)G sequences containing the catalytic Asp residues of the aspartyl protease family are shown in boldface type.

localization of the aspartyl proteases and Kex2 to different cellular compartments (2). Further understanding of the roles of Kex2, Mkc7, and Yap3 proteases in cell growth will be aided by identification both of the relevant substrates and of the cellular pathways in which they function.

Implications for the Roles of Multiple Processing Enzymes in Mammalian Cells. The overlapping functions of the Mkc7, Yap3, and Kex2 proteases raise the question of whether, like Kex2 (3), the yeast GPI-anchored aspartyl proteases also have counterparts in mammalian cells. In fact, aspartyl proteases

Table 2. Proteolytic activity in cells overexpressing Mkc7

Plasmid	Activity, units per fraction					Specific activity in III-S, units/mg
	I	II-P	II-S	III-P	III-S	
pGMKC7	9.7×10^6 (100)	6.4×10^6 (66)	9.0×10^5 (9)	1.3×10^6 (13)	5.3×10^6 (55)	2.3×10^5
pG5	$<1.1 \times 10^4$*	ND	ND	$<9.0 \times 10^2$*	$<5.7 \times 10^3$*	$<2.1 \times 10^2$

Cells [HKY26 (*MATα, yap3*Δ::*LEU2, mkc7*Δ::*HIS3, pep4*::*HIS3, prb1*Δ::*LEU2*) containing pGMKC7 or pG5] were grown to 6×10^7 cells per ml in 1 liter of SDC-uracil, harvested, washed twice in water and once in buffer A [50 mM Hepes, pH 7.5/10 mM EDTA/0.5 mM phenylmethylsulfonyl fluoride/0.1 mM N-tosyl-L-phenylalanine chloromethyl ketone/0.1 mM N^{α}-(*p*-tosyl)lysine chloromethyl ketone/1 mM benzamidine hydrochloride] by centrifugation ($3000 \times g$), and frozen (−80°C). Thawed cells (5 ml, packed) were suspended in buffer A (8 ml), lysed by vortex mixing for 60 20-sec periods with 5 ml of 0.5-mm glass beads (this and subsequent steps were at 0–4°C), and centrifuged, along with washes of beads (3 min, $500 \times g$). The low-speed supernatant fraction (I) was centrifuged (1 h, $100,000 \times g$), producing a supernatant fraction (II-S) and a $100,000 \times g$ pellet (fraction II-P). Fraction II-P was solubilized in 19 ml of buffer A/1% Triton X-100 (30 min on ice) and centrifuged (1 h, $100,000 \times g$) to produce a supernatant fraction (fraction III-S, Triton X-100-solubilized membranes) and a pellet, which was resuspended in buffer A/1% Triton X-100 (fraction III-P). Proteolytic activity was measured. Numbers in parentheses are percent of fraction I. Protein was determined by the BCA assay (Pierce). ND, not determined.

*Activity was not detectable; the signal was <30% of background fluorescence (i.e., <0.3 unit/μl of fraction).

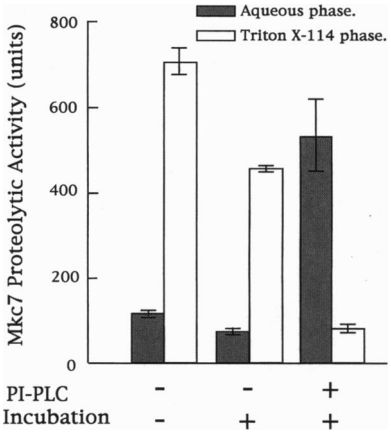

FIG. 3. Effect of PI-PLC treatment on Triton X-114 phase separation of Mkc7 protease activity. Purified Mkc7 protease (2×10^7 units/mg) was subjected to Triton X-114 phase separation (*i*) without treatment, (*ii*) after mock digestion by PI-PLC, or (*iii*) after digestion with 0.05 unit of PI-PLC, and proteolytic activity in each phase was measured. The results shown represent the averages of duplicate assays. Error bars represent differences of the individual measurements from the mean.

have been purified from secretory granule fractions as candidate enzymes for mammalian proopiomelanocortin processing at pairs of basic residues (4) and for maturation of anglerfish prosomatostatin at a single Arg cleavage site (5). Such enzymes could constitute a distinct class of proprotein processing enzymes that would broaden the array of proteolytic enzymes involved in protein biosynthesis in eukaryotes.

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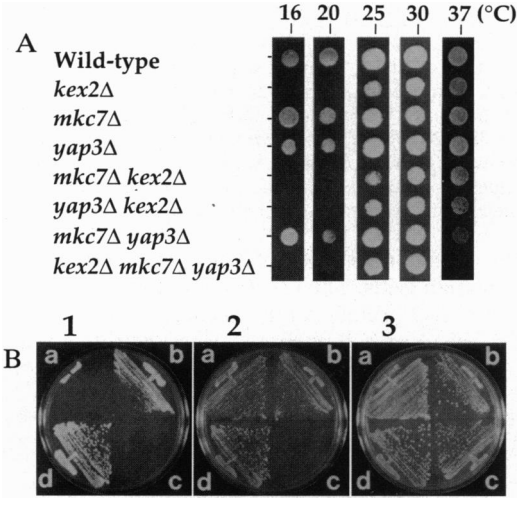

FIG. 4. Synergistic growth effects of *kex2*, *mkc7*, and *yap3* null mutations. (*A*) Logarithmic-phase cells (3×10^7 cells per ml) were grown in YPAD liquid medium at 30°C, diluted 1:4, and spotted with a multiprong inoculator onto YPAD plates. The plates were incubated at the indicated temperatures for 59 h (30°C, 37°C), 70 h (25°C), 82 h (20°C), or 107 h (16°C). Strains: wild-type, CRY2; *kex2Δ*, HKY19; *mkc7Δ*, HKY21; *yap3Δ*, HKY20; *mkc7Δ kex2Δ*, HKY23; *yap3Δ kex2Δ*, HKY22; *mkc7Δ yap3Δ*, HKY24; *kex2Δ mkc7Δ yap3Δ*, HKY25. (*B*) Strains were streaked from single colonies onto YPAD or SDC plates and incubated at 16°C or 30°C. Plates: 1, 6 days on YPAD at 16°C; 2, 6 days on SDC at 16°C; 3, 3 days on SDC at 30°C. Sectors: a, *kex2Δ* (BFY106-4D); b, wild-type (CRY2); c, *kex2Δ mkc7Δ yap3Δ* (HKY25); d, *mkc7Δ yap3Δ* (HKY24).

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