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Isolation and characterization of a yeast gene, *MPD1*, the overexpression of which suppresses inviability caused by protein disulfide isomerase depletion

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Abstract ***MPD1*, a yeast gene the overexpression of which suppresses the inviability caused by the loss of protein disulfide isomerase (PDI) was isolated and characterized. The *MPD1* gene product retained a single disulfide isomerase active site sequence (APWCGHCK), an N-terminal putative signal sequence, and a C-terminal endoplasmic reticulum (ER) retention signal, and was a novel member of the PDI family. The gene product, identified in yeast extract, contained core size carbohydrates. *MPD1* was not essential for growth, but overexpression of the gene suppressed the maturation defect of carboxypeptidase Y caused by *PDI1* deletion, indicative of the related function to PDI in the yeast ER.**

Key words: Protein disulfide isomerase; Multicopy suppressor; *Saccharomyces cerevisiae*

1. Introduction

The endoplasmic reticulum (ER) is the first organella of the secretory pathway. Secretory proteins translated on membrane-bound ribosomes are translocated into the lumen of the ER in a largely unfolded state where they are folded into their native conformation [1], before being transported from the ER [2,3]. It has become increasingly clear that ER proteins function in assisting the folding of secretory proteins. Bip is a so-called molecular chaperon and its function is considered to be to inhibit the aggregation and support the folding and oligomerization of secretory proteins [4]. Protein disulfide isomerase (PDI) is considered to function in the formation of native disulfide bonds [5–7].

Mammalian PDI is a 57 kDa ER protein, and exists as a homodimer. It contains a signal peptide, an ER retention signal (KDEL), and two disulfide isomerase active site sequences (APWCGHCK) [8], functioning independently in disulfide isomerization [9]. Mammalian PDI is also reported to have peptide binding activity [10,11]. Recently, other ER proteins with disulfide isomerase active site sequences, ERp72 [12], P5 protein [13], and PDI isozyme Q-2 [14,15] have been identified. The in vivo functions of these proteins are not yet clear. Günter et al. suggested that they may interact with different sets of target proteins in the ER [16]. On the other hand, Urade et al. reported that ERp72 and Q-2 related protein contain protease activity and suggested that they are involved in the degradation of proteins in the ER [17,18].

PDI from *Sacchromyces cerevisiae* was purified, and was found to have molecular properties very similar to those of mammalian PDIs [19,20]. Molecular cloning of the *PDI1* gene revealed that it encodes a polypeptide of 522 amino acids with 30% identity to mammalian PDIs and that it contains a signal peptide-like sequence, an ER retention signal (HDEL), and two disulfide isomerase active site sequences [21]. *PDI1* is essential for cell growth [21]. It was also cloned and characterized by other groups [22–25]. Yeast PDI also has peptide binding activity [23]. Depletion of PDI was found to cause accumulation of vacuolar protein carboxypeptidase Y (CPY) in a precursor form [24,26,27]. The PDI related gene *EUG1* was cloned by Tachibana et al. [26]. The *EUG1* product (Eug1p) is an ER protein with 43% identity to yeast PDI, but it has only one cysteine per disulfide isomerase active site-like sequence instead of two and seems not to have disulfide isomerase activity [26]. Overproduction of the Eug1p allows cells to grow in the absence of PDI, and partially suppresses the maturation defect of CPY [26].

In the present study, to examine the function of PDI in vivo more extensively, we isolated genes the overexpression of which would suppress the inviability caused by PDI depletion. We isolated a gene and named it *MPD1* (for multicopy suppressor of *PDI1* deletion). It was a novel member of the PDI family with an ER retention signal and only a single disulfide isomerase active site sequence. No homologous region to the peptide binding site of mammalian PDI was observed in the gene product. We also demonstrated that overexpression of the gene partially recover the maturation defect of CPY caused by PDI depletion in vivo.

2. Materials and methods

2.1. Yeast strains, growth media, and materials

S. cerevisiae YPH500 (*MATα ura3 lys2 ade2 trp1 his3 leu2*) [28] and YPH501 (*MATa/MATα ura3/ura3 lys2/lys2 ade2/ade2 trp1/trp1 his3/his3 leu2/leu2*) [28] were used in this study. Growth media were prepared as described [29].

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Abbreviations: PDI, protein disulfide isomerase; ER, endoplasmic reticulum; CPY, carboxypeptidase Y.

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2.2. Production of a galactose-dependent mutant

The *TRP1* gene [30] and the *GAL1* promoter [31] were placed immediately upstream from the open reading frame (ORF) of the chromosomal *PDI1* gene of a haploid YPH500 by homologous recombination. Resulting strains were Trp^+ and showed galactose-dependent growth. One of them was named FW14.

2.3. Cloning of the MPD1 gene

The galactose-dependent mutant FW14 was grown in galactose containing medium, transformed with a yeast genomic library in an *URA3*-containing multicopy plasmid, YEp24, and screened for growth on glucose plates. Plasmids were recovered from viable colonies as described [32], and subjected to Southern blot analysis [33] with a *PDI1*-containing DNA fragment as a probe. One of the clones which did not hybridize with the *PDI1* probe was named pMPD1. The pMPD1 plasmid was partially digested with *Sau*3A1, and cloned into the *Bam*HI site of a *LEU2*-containing multicopy, 2 μm-based vector pYO325 (Y. Ohya, unpublished), to construct mini-libraries. Re-screening was carried out by transforming FW14 with these mini-libraries, to obtain pmMPD1. The insert of pmMPD1 was cloned into a *HIS3*-containing multicopy, 2 μm-based vector pYO323 (Y. Ohya, unpublished), to give pMHis-MPD1.

For confirmation that overexpression of *MPD1* can suppress *PDI1* deletion, a *PDI1/pdi1::LEU2* diploid YT103 was produced from YPH500 as described [21]. YT103 was transformed with pMHis-MPD1, to give YT201. This strain was sporulated and dissected. Leu^+ and His^+ haploid strains were isolated. One of them was named YT201-1B (a *Δpdi1* strain carrying pMHis-MPD1).

2.4. DNA sequencing

DNA sequences of pmMPD1 was determined by the dideoxy-chain termination method using a DNA sequencer (Applied Biosystems) on both strand. The nucleotide and predicted amino acid sequences of the *MPD1* gene was analyzed using Genetyx version 9 (Software Development).

2.5 Disruption of the MPD1 *gene*

Position 117–735 of the *MPD1* gene was replaced with the *URA3* gene [30]. The resulting disrupted copy of the *MPD1* gene was excised out from the plasmid and introduced into YPH501. The resulting strain, YT401, was sporulated and dissected.

2.6. Preparation of antiserum and immunoblot analysis

pmMPD1 was digested with *Hpa*I and *Eco*RI and a 1.3 kb DNA fragment, containing the whole ORF of *MPD1* except the N-terminal region, was recovered and ligated into pGEX-2T [34] digested with *Sma*I and *Eco*RI. The plasmid was introduced into *E. coli* and the glutathione *S*-transferase-Mpd1p fusion protein was induced as described [34]. Since the fusion protein was insoluble, it was purified by SDS-PAGE and electroelution and used to immunize New Zealand White rabbits.

Cell lysates were prepared using glass beads. Endoglycosidase H (Endo H) treatment was performed as described [20]. Immunoblot analysis was performed by a standard protocol.

2.7. Pulse-chase experiment

Pulse-chase experiments were performed as described by Rothblatt et al. [35]. Yeast strains YPH500, FW14, FW14 bearing pMHis-MPD1 were grown to the stationary phase in galactose containing medium, and then inoculated and cultured in 50 ml of synthetic minimal medium containing 5% glucose for 16 h. Cells were harvested, resuspended to 2 OD_{600} units/ml, labeled with ^{35}S-labelling mix (Amersham) for 10 min, and chased for 0, 5, 15, 60 min. Immunoprecipitation was performed using anti-CPY antibody (kindly provided by T. Oka and A. Nakano) and immunoprecipitates were analyzed by SDS-PAGE and autoradiography.

3. Results

3.1. Isolation of multicopy suppressor genes of PDI1 *deletion*

The *PDI1* gene of *S. cerevisiae* is essential for cell growth [21]. Multicopy suppressors of *PDI1* deletion could encode proteins that either substitute for or bypass its essential function. Therefore, screening was performed using a conditional mutant. A mutant strain (FW14) that was able to grow on galactose containing medium, but was unable to grow on glucose containing

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TTATCTGGATACAAATTGACGCAAATTTCTCTTTATATTCCTCCTTTGTCGCCCAGTGTCATGTGCTTATCCCTTGAAAAAAGGGCCGTTGTTCGAGCCTTGCGAGGCGTATAAAAATTT -121
GGTAAAAATTAAAATTGACACGTAAAAGTTAAATAACATGAAGAGAAATATCTTTTCGCAGTCATTTTCAACAGTAAGCATCCACTTAACACAATTAGGAGAGACAAAATTTGACATATA   -1

ATGTTATTTCTTAATATTATTAAGCTCCTTTTGGGACTTTTTATTATGAATGAAGTAAAGGCGCAAAACTTTTACGATTCCGATCCTCATATATCAGAGTTAACGCCAAAAAGCTTCGAT  120
M  L  F  L  N  I  I  K  L  L  L  G  L  F  I  M  N  E  V  K  A  Q  N  F  Y  D  S  D  P  H  I  S  E  L  T  P  K  S  F  D

AAAGCGATCCATAACACAAATTACACATCATTAGTGGAATTTTATGCCCCGTGGTGCGGCCATTGTAAGAAGCTCTCTAGTACGTTCCGCAAGGCAGCAAAAAGATTGGATGGTGTAGTC  240
K  A  I  H  N  T  N  Y  T  S  L  V  E  F  Y  A  P  W  C  G  H  C  K  K  L  S  S  T  F  R  K  A  A  K  R  L  D  G  V  V

CAAGTTGCTGCTGTAAACTGTGACCTTAACAAGAATAAGGCTTTGTGTGCTAAATACGACGTAAACGGATTTCCCACGTTAATGGTATTTAGGCCCCCAAAAATTGACCTATCTAAGCCA  360
Q  V  A  A  V  N  C  D  L  N  K  N  K  A  L  C  A  K  Y  D  V  N  G  F  P  T  L  M  V  F  R  P  P  K  I  D  L  S  K  P

ATAGATAACGCCAAAAAAAGTTTCAGCGCTCATGCCAATGAAGTGTACTCAGGTGCAAGAACTCTCGCGCCTATTGTTGATTTTTCTCTTTCAAGAATAAGGTCATATGTCAAAAAGTTT  480
I  D  N  A  K  K  S  F  S  A  H  A  N  E  V  Y  S  G  A  R  T  L  A  P  I  V  D  F  S  L  S  R  I  R  S  Y  V  K  K  F

GTCCGTATAGATACACTTGGCTCTTTACTTAGAAAGTCACCCAAACTTTCCGTGGTGTTGTTTTCCAAACAAGACAAAATTTCACCGGTTTATAAAAGCATTGCCCTTGATTGGTTAGGA  600
V  R  I  D  T  L  G  S  L  L  R  K  S  P  K  L  S  V  V  L  F  S  K  Q  D  K  I  S  F  V  Y  K  S  I  A  L  D  W  L  G

AAGTTCGATTTTTATTCAATTTCAAACAAAAAACTCAAGCAACTAACCGATATGAACCCAACATATGAAAAAACTCCTGAGATTTTCAAATATTTGCAGAAGGTCATTCCTGAACAGCGA  720
K  F  D  F  Y  S  I  S  N  K  K  L  K  Q  L  T  D  M  N  P  T  Y  E  K  T  P  E  I  F  K  Y  L  Q  K  V  I  P  E  Q  R

CAGAGCGATAAAAGTAAGCTTGTCGTTTTTGATGCAGACAAAGATAAATTTTGGGAGTATGAAGGGAACTCAATCAACAAAAATGACATTTCCAAATTTCTGCGGGACACTTTTAGTATT  840
Q  S  D  K  S  K  L  V  V  F  D  A  D  K  D  K  F  W  E  Y  E  G  N  S  I  N  K  N  D  I  S  K  F  L  R  D  T  F  S  I

ACCCCCAATGAGGGTCCTTTTAGTAGACGTTCTGAATATATTGCTTACTTAAAAACTGGCAAGAAGCCAATTAAAAAGAACCATTCCTCCTCAGGAAACAAGCACGACGAATTGTAGTGG  960
T  P  N  E  G  P  F  S  R  R  S  E  Y  I  A  Y  L  K  T  G  K  K  P  I  K  K  N  H  S  S  S  G  N  K  H  D  E  L  *

TACGTTTCCTCATTCAATGATTATCTAATTAAACACAACATATGTATCTATATAGTCCACTAACTGATGGCATGGACGTTTACTTTTATTTGCAATAATAACTTTTATCTGAGCGGTTAA 1080
ATACCGAAAGACACGTTTATTACCCGCTTTGAAAATTTTCAACCGATGAGCTTGCCTTTCTACCGGAAAACTGGGGCATAAGAATAAGTACTAAATAGTATAATTAAATTGCCATCCAAA 1200
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Fig. 1. The nucleotide sequence and the deduced amino acid sequence of the *MPD1* (A) gene. The N-terminal putative signal sequence and the C-terminal ER retention signal are underlined. The disulfide isomerase active site sequence is boxed. Putative glycosylation sites are double underlined. The TATA box and region homologous to the unfolded protein response element (UPR) [34] are indicated by dashed underline. The nucleotide sequence data will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the following accession number: D34633.

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Mpd1p 27 SDPHISELTPKSFDKAIHNTNYTS--LVEFYAPWCGHCKKLSSTFRKAAKRL-D-GVVQVAAVNCDLNKNKALCAKYDVNGFPTLMVFRP 112
Q-2   23 AASDVLELTDENFESRVSDTGSAGLMLVEFFAPWCGHCKRLAPEYEAAATRL-K--IVPLAKVDC--TANTNTCNKYGVSGYPTLKIFRA 117
yPDI  30 EDSAVVKLATDSFNEYIQSHDLV---LAEFFAPWCGHCKNMAPEYVKAAETLVE-KNITLAQIDC--TENQDLCMEHNIPGFPSLKIFKN 112
rPDI  24 EEDNVLVLKKSNFAEALAAHNYL---LVEFYAPWCGHCKALAPEYAKAAAKL-KAEGSEIRLAKVDATEESDLAQQYGVRGYPTIKFFKN 109
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Fig. 2. Alignment of deduced amino acid sequence of the *MPD1* gene with PDIs and the PDI isozyme Q-2. Identical amino acid residues are underlined. The abbreviations; Q-2, PDI isozyme Q-2; yPDI, yeast PDI; rPDI, rat PDI.

medium because of the depletion of PDI was transformed with a yeast genomic library constructed with YEp24 multicopy vector. Thirteen transformants that could grow on glucose containing medium were obtained. Plasmids were recovered from these transformants and subjected to restriction endonuclease mapping and Southern blot analysis. Nine contained the *PDI1* gene, two contained no insert, and two contained inserts differing from the *PDI1* gene. The genes in the two latter plasmids were designated as *MPD1* and *MPD2*. Herein, we report the analysis of the *MPD1* gene. Since the plasmid contained an insert of about 9 kb, it was partially digested with *Sau*3AI and a 1.8 kb DNA fragment with suppressor activity was recovered.

To confirm that the *MPD1* gene can suppress *PDI1* deletion, we transformed the diploid strain YT103 (*PDI1/pdi1::LEU2*) with pMHis-MPD1, a multicopy plasmid containing the *MPD1* gene (*HIS3* marker). His$^+$ transformants were sporulated and tetrads were dissected. Since *PDI1* is essential, no Leu$^+$ colony was generated from untransformed YT103. By contrast, Leu$^+$/His$^+$ colonies were generated from spores of transformed diploid strain, YT201 (transformed with pMHis$^+$-MPD1). Leu$^+$/His$^+$ progenies grew as well as the wild type cells indicating that overexpression of *MPD1* can suppress *PDI1* deletion.

3.2. *Protein encoded by* MPD1 *is a novel member of the PDI family*

The nucleotide sequence of *MPD1* revealed an ORF of 954 nucleotides coding for a polypeptide of 318 amino acids (Fig. 1). The *MPD1* gene product (Mpd1p) contained an N-terminal signal peptide-like sequence and a C-terminal ER retention signal of *S. cerevisiae* (HDEL), suggesting that it is an ER protein. It also contained a single sequence, APWCGHCK, which is identical to the active site sequences of mammalian and yeast PDIs [8,21]. Three consensus sequences for N-linked glycosylation were found. Mpd1p was most similar to the region containing the first disulfide isomerase active site of the PDI isozyme Q-2 (42% identity in 88 amino acids) [15] (Fig. 2). Therefore, Mpd1p is a novel member of the PDI family. C-terminal half was not homologous with any protein in data bases. The protein did not have any region homologous with the peptide binding region of mammalian PDIs [11].

In the promoter region, we found a TATA box and a sequence related to the unfolded protein responsive element which is necessary for induction in response to accumulation of unfolded proteins in the ER [26,36].

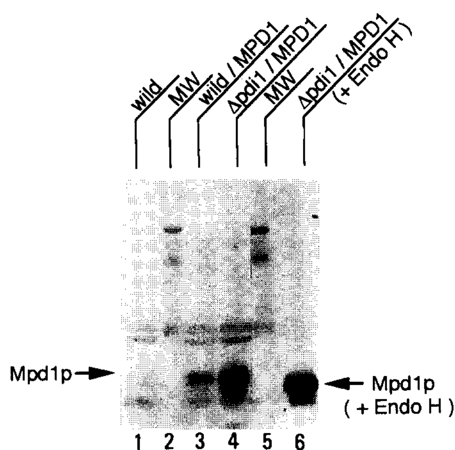

Fig. 3. Immunoblot analysis of Mpd1p. Extracts were prepared from YPH500 (wild type strain; lanes 1), YT201-1A (wild type strain bearing pMHis$^+$-MPD1; lane 3), YT201-1B (*Δ pdi1* strain bearing pMHis$^+$-MPD1; lanes 4 and 6). For lane 6, deglycosylation by endoH treatment was performed. Immunoblot analysis was performed using the anti-Mpd1p antibody. Lanes 2 and 5 are molecular weight markers.

3.3. MPD1 *is not essential for growth and is expressed at low levels in wild type cells*

Null mutation of *MPD1* was produced by one step gene disruption. The *MPD1* gene was mutated by replacement with *URA3*, and used to transform diploid cells (YPH501). Chromosomal deletion of the *MPD1* gene of transformants was confirmed by Southern blotting. After sporulation, spores were dissected and haploid segregants were scored for viability. Four viable spores were often observed and the *Δ-mpd1* haploid strain grew as well as the wild type strain. These findings indicate that *MPD1* is not essential for cell growth.

To characterize Mpd1p, yeast cell extracts were subjected to SDS PAGE and analysed by Western blotting using an anti-Mpd1p antibody (Fig. 3). Mpd1p could not be detected in a wild type cell extract, but was detected in an extract of cells carrying multicopy plasmids containing *MPD1*, and was more abundant in an extract of cells depleted of PDI and carrying this plasmid. Its molecular size was 36 kDa, and was shifted to 31 kDa by endoglycosidase H (endo H) treatment. Since the size of one core-oligosaccharide is about 2 kDa, this protein presumably has two or three core-oligosaccharides. These findings were consistent with the molecular size and three *N*-glycosylation sites deduced from our sequence data. Northern blot analysis revealed that *MPD1* is expressed in wild type cells (data not shown).

3.4. *Overproduction of Mpd1p in cells depleted of PDI partially restores the rate of CPY maturation*

Depletion of PDI has been reported to cause accumulation of the vacuolar protein, CPY, in an early secretory pathway precursor form [26,27]. Since overproduction of Mpd1p allowed cells to grow in the absence of PDI, using pulse chase analysis, we examined whether the maturation of CPY is normal in these cells (Fig. 4). In wild type cells, most of the CPY became the mature form within 15 min. As reported, when the galactose dependent mutant (FW14) was preincubated in glucose containing medium for 16 h, most of the CPY remained

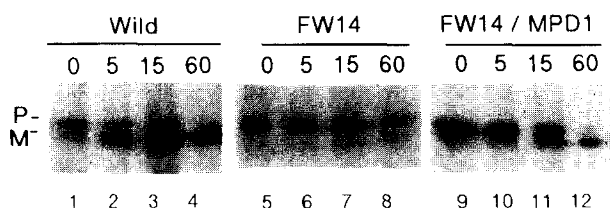

Fig. 4. Functional complementation of PDI depletion by overproduction of Mpd1p. CPY was immunoprecipitated and analysed. P is the precursor form and M is the mature form of CPY. Cells grown in galactose-containing medium were inoculated into glucose-containing medium and cultured for 18 hours. Then the cells were labelled for 10 min, and chased for 0 (lanes 1, 5, 9), 5 (lanes 2, 6, 10), 15 (lanes 3, 7, 11), or 60 (lanes 4, 8, 12) min. Lanes 1–4 = YPH500 (wild type); lanes 5–8 = FW14 (the galactose-dependent mutant); lanes 9–12 = FW14 transformed with pMHis$^+$-MPD1.

in the precursor form. In FW14 bearing pMHis$^+$-MPD1, in the same condition, approximately 80% of the CPY became the mature form in 60 minutes, These findings indicated that the overproduction of Mpd1p in cells depleted of PDI partially restores the rate of CPY maturation, and that the protein has a function related to PDI.

4. Discussion

We have isolated a novel yeast gene, *MPD1*, the overexpression of which suppresses the inviability caused by *PDI1* deletion. The protein encoded by *MPD1* is a novel member of PDI family. The protein contained an N-terminal putative signal peptide, a C-terminal ER retention signal (HDEL), and a single disulfide isomerase active site sequence (APWCGHCK). Mpd1p must exhibit a function similar to PDI, because overexpression of the protein can compensate for the loss of PDI, and because it is structurally related to PDI. Mpd1p was most similar to the region containing the first disulfide isomerase active site of the PDI isozyme Q-2 [15]. Q-2 related protein is reported to have protease activity [17], so, we are interested in whether Mpd1p has protease activity like Q-2 related protein. The C-terminal half of Mpd1p was not homologous to any other proteins in a search of the database. Function of the C-terminal half is unknown. Mpd1p must be an ER protein because: (i) it contains a putative signal peptide and a ER retention signal; (ii) its overproduction suppresses the growth defect caused by depletion of PDI which functions in the ER; and (iii) it contains core size carbohydrates.

Mori et al. reported that a 22 bp *cis*-acting element, UPR (GGAACTGGACAGCGTGTCGAAA), is necessary and sufficient for induction of the yeast *KAR2* (Bip) gene by unfolded proteins [36]. The *EUG1* gene has also been reported to be induced by unfolded proteins, and its promoter region contains the sequence, GGCACGCGTG [26]. In the promoter region of the *MPD1* gene, we found a sequence, GCCTTGCGAG, that resembles these sequences. The fact that Mpd1p is more abundant in a strain depleted of PDI than in a strain containing PDI may be explained by the presence of UPR like sequence and by accumulation of precursor form or unfolded form of secretory proteins in the ER [37].

LaMantia et al. reported that the disulfide isomerase activity of PDI is not essential for cell growth [27] and suggested that the essential function of PDI may be its peptide binding activity. In this work, depletion of PDI was complemented by overproduction of Mpd1p, which has a disulfide isomerase active site, but has no region homologous to the peptide binding site. Our findings suggest that disulfide isomerase activity may be important. We must clarify whether the complementation by Mpd1p is dependent on its disulfide isomerase activity and whether the essential function of PDI is its disulfide isomerase activity. A mutagenesis study is underway.

In the yeast ER, there exist proteins belonging to PDI family, PDI, Eug1p, and Mpd1p. We are now analysing the *MPD2* gene, which is the second multicopy suppressor gene of *PDI1* deletion, we have isolated. Relationships of PDI, Eug1p, Mpd1p and other proteins in the yeast ER must be complex and important. Further studies are needed to clarify the functional relationship and functional difference of these proteins in disulfide bond formation and folding of secretory proteins.

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