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Short Communication

Protein Disulfide Isomerase in Spore Germination and Cell Division

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Protein disulfide isomerase (PDI) is a protein of the endoplasmic reticulum (ER) that is essential for the unscrambling of nonnative disulfide bonds. Here, we have determined the importance of PDI to both spore germination and vegetative cell division. To vary the concentration of PDI in the ER, we used plasmids that direct the expression of rat PDI fused at its N terminus to either the α-factor pre-pro segment or the α-factor pre sequence, and fused at its C terminus to either the mammalian (KDEL) or the yeast (HDEL) ER retention signal. Classical yeast genetic (tetrad) analyses, and plasmid loss and plasmid shuffling experiments were used to evaluate the ability of these constructs to complement haploid *Saccharomyces cerevisiae* cells in which the endogenous *PDI1* gene had been deleted. We find that basal levels of PDI in the ER are sufficient for vegetative growth. In contrast, high levels of PDI in the ER are required for efficient spore germination. Thus, catalysis of the unscrambling of nonnative disulfide bonds in cellular proteins is more important during spore germination than during vegetative cell division.
Key words: Cell division / Endoplasmic reticulum / Protein disulfide isomerase / *Saccharomyces cerevisiae* / Spore germination.

A protein disulfide isomerase (PDI) activity was predicted to exist before the enzyme itself was isolated (Goldberger *et al.*, 1963; Venetianer and Straub, 1963). The observed contrast between the slow formation of native disulfide bonds *in vitro* and their apparently rapid formation during protein biosynthesis indicated that the process was catalyzed by an enzyme. Subsequently, PDI was found to be an abundant protein of the endoplasmic reticulum (ER), the cellular compartment in which disulfide bonds are most often formed in eukaryotic cells.

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In vitro, PDI catalyzes the oxidation of dithiols to form disulfide bonds, and the reduction and isomerization of existing disulfide bonds (Hu and Tsou, 1991; Freedman *et al.*, 1994). The cDNA sequence that codes for PDI has been determined for several species (Edman *et al.*, 1985; Morris and Varandani, 1988; Scherens et al., 1991). The mammalian protein contains two active sites of sequence WCGHCK, which act independently (Vuori *et al.*, 1992a). The C terminus of mammalian PDI ends with the tetrapeptide KDEL, which has been implicated as the signal for retention of a protein in the ER of mammalian cells (Munro and Pelham, 1987). The sequence of the cDNA that codes for *Saccharomyces cerevisiae* PDI has also been determined (Farquhar *et al.*, 1991; LaMantia *et al.*, 1991; Scherens *et al.*, 1991; Tachikawa *et al.*, 1991). The amino acid sequence contains an N-terminal signal peptide, two putative active sites, five putative N-glycosylation sites, and a C terminus ending with HDEL, the *S. cerevisiae* equivalent of KDEL (Pelham *et al.*, 1988; Scherens *et al.*, 1991). Although the 522 amino acid residues of the encoded protein share only approximately 30% identity to mammalian PDI's, the active-site sequences are conserved completely.

The *PDI1* gene is essential for the viability of *S. cerevisiae* (Farquhar *et al.*, 1991; LaMantia *et al.*, 1991; Scherens *et al.*, 1991; Tachikawa *et al.*, 1991). PDI can be replaced *in vivo* by homologs ERp72 (Günther *et al.*, 1993), and variants of thioredoxin (Chivers *et al.*, 1996). Recently, we described an *S. cerevisiae* expression system for PDI that allows for the study of protein structure – function relationships *in vitro* and *in vivo* (Laboissière *et al.*, 1995a). We used this system to demonstrate that the essential function of PDI in *S. cerevisiae* is to unscramble nonnative disulfide bonds in the ER (Laboissière *et al.*, 1995b). Here, we analyze the ability of four rat PDI constructs to complement a null mutation of the *S. cerevisiae PDI1* gene. These constructs vary in their abilities to target PDI to the ER, and to retain it there.

To determine the role of PDI in *S. cerevisiae* growth, plasmids that direct the expression of rat PDI were transformed into *S. cerevisiae* cells in which the endogenous *PDI1* gene had been deleted (*pdi1Δ*). Suppression of the mutant phenotype by the production of rat PDI was assessed in three ways. First, we determined the ability of plasmids encoding rat PDI to rescue haploid spores that had inherited the *pdi1Δ::HIS3* allele. Second, we monitored the ability of *pdi1Δ*/haploid cells to lose an essential episomal copy of the yeast *PDI1* gene on pCT37 (Table 1) by the alternative retention of plasmids that direct the ex-

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Table 1 Plasmids Used in This Study.

The construction of plasmids pMAL3.1, pMAL5.1, pMAL6.1, and pMAL7.1, which direct the production of rat PDI, under the control of the ADH2-GAPDH promoter, was described previously (Laboissière *et al.*, 1995a). A plasmid, pMAL9, that directs the production of *S. cerevisiae* PDI under the control of its own promoter was constructed as follows. A fragment containing the cDNA that codes for *S. cerevisiae* PDI was isolated from plasmid pFL44 *Bam*HI-*Bam*HI by digestion with *Apa*I and *Pst*I, and was inserted into plasmid pRS424 that had been digested with *Apa*I and *Pst*I. pCT37 is a *URA3* plasmid that encodes yeast PDI under the control of a GAL promoter (Tachibana and Stevens, 1992).

		Encoded protein disulfide isomerase		
Plasmid	Marker	Species	N terminus	C terminus
pMAL3.1	TRP1	rat	α-factor pre-pro segment	KDEL
pMAL5.1	TRP1	rat	α-factor pre-pro segment	HDEL
pMAL6.1	TRP1	rat	α-factor pre-sequence	KDEL
pMAL7.1	TRP1	rat	α-factor pre-sequence	HDEL
pMAL9	TRP1	*S. cerevisiae*	endogenous signal sequence	HDEL
pCT37	URA3	*S. cerevisiae*	endogenous signal sequence	HDEL

Table 2 Tetrad Analysis of *PDI1/pdi1Δ::HIS3* Cells Transformed with Plasmids Encoding Rat PDI.

		Segregation of spores (viable:nonviable)					Viable spores	
Plasmid	Tetrads	4:0	3:1	2:2	1:3	0:4	His^+/Trp^+	His^+/Trp^-
pMAL3.1	71	0	0	45	20	6	0	0
pMAL5.1	70	2	4	50	9	5	9[a]	0
pMAL6.1	77	0	0	51	23	3	0	0
pMAL7.1	85	0	0	43	38	4	0	0
pMAL9	24	15	6	2	1	0	35	0

[a] One of the 3:1 tetrads had 2 His^+ and 1 His^- spore.
Method is as described in Figure 1.

pression of rat PDI variants (non-selective plasmid loss). Third, we monitored the rate of growth of *pdi1Δ*/pCT37 cells containing plasmids that direct the expression of rat PDI under conditions that forced the loss of pCT37 (plasmid shuffling).

Plasmids encoding *S. cerevisiae* (pMAL9 or pCT37) or rat PDI(pMAL3.1, pMAL5.1, pMAL6.1 or pMAL7.1) were transformed into a heterozygous *PDI1/pdi1Δ::HIS3* diploid strain. After dissection of tetrad asci and germination at 30 °C on solid medium containing dextrose (2.0% w/v), the haploid progeny of these transformants were analyzed for spore viability, segregation of the *pdi1Δ::HIS3* disruption allele (as indicated by histidine prototrophy, His^+), segregation of the plasmid encoding rat PDI (as indicated by tryptophan prototrophy, Trp^+), and spore mating type.

Only the plasmids encoding *S. cerevisiae* PDI (pMAL9) or rat PDI fused to the α-factor pre-pro segment and containing the yeast retention signal HDEL (pMAL5.1), and grown at 30 °C on medium containing 2% (w/v) dextrose were able to rescue the PDI deficiency (Table 2). Tetrads complemented with *S. cerevisiae* PDI grew within 3 days. In tetrads complemented with this construct, 2 spores per tetrad that were His^- (and thus *PDI1* at the chromosomal locus) grew within 3 days (Figure 1). An additional 1 or 2 spores became visible only after 4 –7 days, and gave rise to slowly growing colonies that were Trp^+ (pMAL5.1) and His^+ (*pdi1Δ::HIS3*). Similar experiments were done in which after dissection of tetrad asci, spores were germinated at 15 or 37 °C on solid medium containing 2.0% (w/v) dextrose or at 30 °C on solid medium containing 0.5 or 1.0% (w/v) dextrose. No complementation was observed under these conditions (results not shown). No difference in the production of rat PDI by the diploid parents was observed by immunoblot analysis (data not shown).

We monitored *pdi1Δ*/pCT37 cells (which were haploid cells that express *S. cerevisiae* PDI from plasmid pCT37 rather than from a chromosome) containing plasmids that direct the expression of rat PDI for the loss of pCT37. Haploid *pdi1Δ* cells complemented by *S. cerevisiae* PDI were obtained by transforming *PDI1/pdi1Δ::HIS3* with pCT37, sporulating the transformants, dissecting the segregants, and isolating His^+/Ura^+ colonies. *pdi1Δ*/pCT37 haploid cells were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9. Transformants were selected on solid medium deficient in tryptophan but containing uracil. Single isolates were grown in liquid medium lacking only tryptophan to allow cells to lose pCT37 while forcing them to retain their *TRP1* plasmid. After several rounds of growth, cells were grown on tryptophan dropout plates to isolate single colonies. These colonies were grown on tryptophan dropout plates, and then replica plated onto tryptophan and uracil dropout plates to check for the presence of the *TRP1* and *URA3* plasmids. Table 3 shows that cells containing pMAL5.1 (rat PDI fused to the α-factor pre-pro segment and HDEL) often lost pCT37. In contrast, this loss was infrequent for cells containing pMAL3.1 (KDEL), and never occurred in cells containing plasmids pMAL6.1 or pMAL7.1 (α-factor pre sequence).

In a related approach, we monitored the growth of *pdi1Δ*/pCT37 cells containing plasmids that direct the expression of rat PDI under conditions that forced the loss of pCT37. Transformants were cultured and grown on plates

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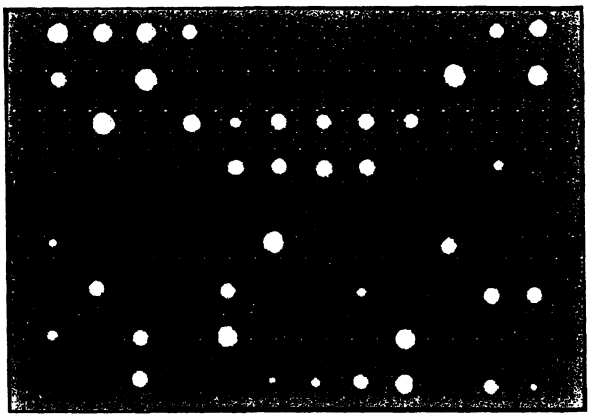

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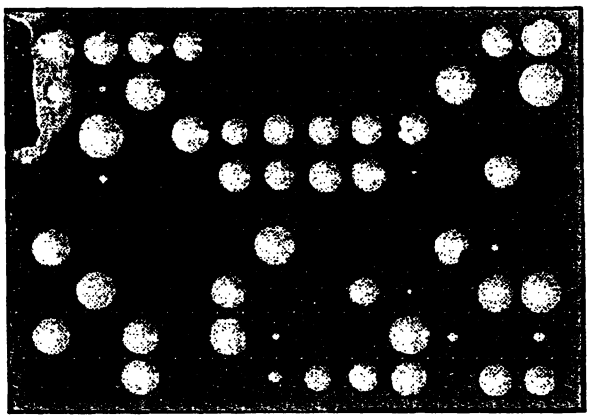

Fig. 1 Germination of Haploid *pdi1Δ S. cerevisiae* Spores Complemented with Rat *PDI*.
Plates were photographed (A) 3 days or (B) 7 days after dissection of tetrads.
Methods: Diploid yeast strain YPH274 α/a *pdi1Δ::HIS3 (ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1)* was transformed with plasmid pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9. Transformants were selected on tryptophan dropout plates. Sporulation was induced by growth on potassium acetate plates (1% w/v potassium acetate, 0.1% w/v yeast extract, 0.05% w/v dextrose, 2% w/v agar), and the resulting tetrads were dissected by micromanipulation onto solid medium containing dextrose (2.0% w/v) and incubated at 30 °C, as described (Shermann, 1991). Spores obtained from tetrad dissection were analyzed by growth on solid tryptophan, uracil, or histidine dropout medium. Mating types of strains were determined by their ability to mate with haploid strains 125 (a *hom3*) and 126 (α *hom3 ilv*). Mating types of complementants were determined as follows. Trp^+ Ura^- cells were grown on solid YEPD medium and mated with cells from strain YPH252 (α *ura3-52 lys2-801 ade2-101 trp1-Δ1 his3-Δ200 leu2-Δ1*). After 24 h, the resulting cells were streaked for single colonies onto tryptophan dropout plates. Single colonies were picked, cultured, and mated for 24 h with haploid strain 125 or 126, and then replica plated onto minimal plates to select for diploids. Colonies that did not mate were considered diploids. Cells from these non-mating colonies were sporulated on potassium acetate plates. Tetrads were dissected and analyzed as described above.

Table 3 Loss of Plasmid Encoding *S. cerevisiae* PDI from Cells Producing Rat PDI.[1]

Plasmid	Total colonies	Trp^+/Ura^- colonies	Plasmid loss (%)
pMAL3.1	90	3	3.3
pMAL5.1	306	146	48
pMAL6.1	249	0	0
pMAL7.1	229	0	0
pMAL9	208	14	6.7

[1] Data were from 4 or 5 independent cultures.
Strain YPH 274α/a:Δ*pdi::HIS3* was transformed with plasmid pCT37. Transformants were selected on solid uracil dropout medium. Sporulation was induced and tetrads dissected onto solid YEP medium containing galactose (2% w/v) and raffinose (1% w/v). *pdi1Δ* haploids complemented by yeast PDI (*pdi1Δ*/pCT37) were identified by growth on histidine and uracil dropout plates. *pdi1Δ*/pCT37 haploids were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, or pMAL7.1. Transformants were selected by growth on uracil/tryptophan dropout plates containing galactose (2% w/v) and raffinose (1% w/v). Transformants were transferred to tryptophan dropout medium containing dextrose (1% w/v). The resulting culture was allowed to grow for 24 h, and then diluted so that $A = 0.10$ at 600 nm. The dilution/growth cycle was repeated, and aliquots of the resulting culture were grown on solid tryptophan dropout medium containing dextrose (1% w/v). The resulting colonies were replica plated onto tryptophan or uracil dropout plates.

containing 5-FOA, which selects for uracil auxotrophs (Sikorski and Boeke, 1991) and thus forces *pdi1Δ*/pCT37 cells to lose pCT37 in order to grow. Under these conditions only cells that produce rat PDI form colonies. Cells containing plasmids that encode any variant of rat PDI were able to form colonies, though at different times after plating on 5-FOA medium. Cells containing plasmids pMAL3.1 or pMAL5.1 (α-factor pre-pro segment) formed colonies within 2–3 days. Cells containing pMAL7.1 (pre α-factor sequence and HDEL) formed colonies in 5–6 days. Cells containing plasmid pMAL6.1 (α-factor pre sequence and KDEL) formed colonies after 8 days. Colonies on the 5-FOA plates were prototrophic for histidine and tryptophan (His^+ Trp^+) but auxotrophic for uracil (Ura^-), which indicated the presence of the *pdi1Δ::HIS3* allele, a plasmid encoding rat PDI, and the loss of plasmid pCT37.

Combined, the results from the plasmid loss and plasmid shuffling experiments indicate that rat PDI fused to the α-factor pre-pro segment and HDEL retention motif complemented *pdi1Δ S. cerevisiae* cells. Rat PDI fused to the α-factor pre-pro segment and KDEL retention motif was also able to complement, but with lower efficiency. Finally, plasmids encoding rat PDI fused to the α-factor pre sequence were poor in their complementation abilities. The analysis of the subcellular location of rat PDI from these constructs before plasmid shuffling demonstrated that

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the α-factor pre sequence was relatively ineffective in directing PDI to the ER (Figure 2), leaving it in the cytosol (results not shown).

To quantify the ability of our different rat PDI constructs to complement *pdi1Δ S. cerevisiae*, we grew the Trp^+ Ura^- cells that resulted from plasmid shuffling in liquid medium containing 1% or 8% (w/v) dextrose [YEP(1%)D and YEP(8%)D media] and measured their doubling times (Table 4). All cells complemented with rat PDI grew more slowly than did those complemented with yeast PDI. Cells containing pre-pro α-factor~PDI~KDEL or pre-pro α-factor~PDI~HDEL had doubling times that were indistinguishable from each other and significantly lower than those of cells containing pre α-factor~PDI~KDEL or pre α-factor~PDI~HDEL. These results are in gratifying agreement with the results obtained by plasmid shuffling. Although growth in the presence of a high concentration of glucose represses expression from the *ADH2–GAPDH* promoter that controls the production of rat PDI, no significant difference in cell growth at 1 and 8% (w/v) glucose was detectable.

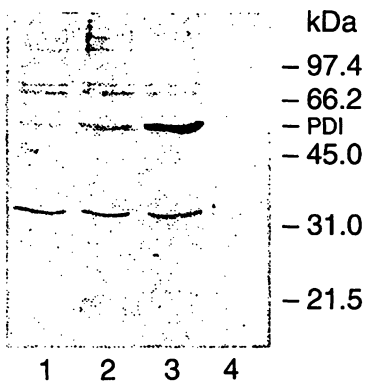

Fig. 2 Protein Transport to the ER Is Inefficient when Directed by the pre α-Factor Sequence.
Immunoblot of microsomal protein from *S. cerevisiae* strain *pdi1Δ*/pCT37 transformed with plasmids that direct the production of rat PDI. Each lane contains the same amount of total protein. Blot was probed with polyclonal antibodies against bovine PDI. Lane 1: pMAL6.1 (pre α-factor~PDI~KDEL); lane 2: pMAL7.1 (pre α-factor~PDI~HDEL); lane 3: pMAL5.1 (pre-pro α-factor~PDI~HDEL); lane 4: pMAL9 (*S. cerevisiae* PDI).
Methods: Chicken polyclonal antibodies against bovine PDI were obtained as described (Polson *et al.*, 1980), and used at a dilution of 1:500. This antibody cross-reacts with rat PDI but not yeast PDI (Laboissière *et al.*, 1995a), which is glycosylated extensively. Cultures (50 ml) of *S. cerevisiae* were grown at 30 °C until $A = 0.5 - 0.8$ at 600 nm. The cells were collected by centrifugation, washed with distilled water (20 ml), and resuspended in 0.10 M Tris-H_2SO_4 buffer, pH 9.4, containing DTT (20 mM) such that $A = 20$. This suspension was incubated for 10 min at 30 °C. Cells were then washed in aqueous sorbitol (1.2 M) and resuspended in spheroplasting buffer, which was 10 mM potassium phosphate buffer, pH 7.2, containing sorbitol (1.2 M) and DTT (2.0 mM), such that $A = 50$. Cells were converted into spheroplasts by adding Novozym 234 from Novo BioLabs (Bagsvaerd, Denmark) to a final concentration of 3 mg/ml, and incubating the resulting solution at 30 °C for 30 min. Spheroplasts were washed twice with two volumes of spheroplasting buffer. Spheroplast lysis and protein isolation were then performed as described (Bostian *et al.*, 1983) to produce three fractions: particulate, microsomal (which contains the contents of the ER), and soluble (which contains the contents of the cytosol). The microsomal and soluble fractions were subjected to denaturing gel electrophoresis as described (Ausubel *et al.*, 1989). Immunoblotting was performed as described (Burnette, 1981), and analyzed using an ECL kit from Amersham Life Science (Arlington Heights, IL) according to the manufacturer's instructions.

Table 4 Doubling Times of Haploid *pdi1Δ S. cerevisiae* Cells Complemented with Plasmids Encoding Rat PDI.

	Medium	
Plasmid	1% Dextrose	8% Dextrose
pMAL3.1	1.7 ± 0.3	1.8 ± 0.2
pMAL5.1	1.8 ± 0.2	1.7 ± 0.3
pMAL6.1	3.4 ± 0.6	nd
pMAL7.1	2.8 ± 0.3	nd
pMAL9	1.0	1.0

nd, not determined.
pdi1Δ/pCT37 haploids were transformed with plasmids pMAL3.1, pMAL5.1, pMAL6.1, pMAL7.1, or pMAL9 and then grown on tryptophan dropout plates. Colonies were replica plated onto plates containing 5-fluoroorotic acid (5-FOA, 1 mg/ml) (Sikorski and Boeke, 1991). Once growth was observed (2 – 5 days), colonies were replica plated onto tryptophan and uracil dropout plates and onto plates containing 5-FOA, to repeat the selection cycle. Trp^+/Ura^- colonies were isolated from the second round of selection. Haploid *pdi1Δ* cells complemented with rat or yeast PDI were inoculated into YEP(1%)D or YEP(8%)D medium such that $A = 0.025$ at 600 nm (10^6 cells/ml). The resulting cultures were grown at 30 °C. At 2-h intervals, an aliquot was removed and its *A* at 600 nm was recorded. Log *A* was plotted versus time, and the slope of the linear portion of the curve was determined by linear least squares analysis. Doubling time was calculated by dividing log 2 by the slope of the curve. At least 5 different clones from each construct were analyzed. The mean and standard deviation of the doubling times for cells containing each construct were calculated. Growth rates were normalized to the growth rate of wild-type cells by dividing the doubling time of haploid *pdi1Δ* cells complemented with a rat *PDI* construct by the doubling time of haploid *pdi1Δ* cells complemented with pMAL9.

To determine if germination depends on PDI, we mated haploid *pdi1Δ* cells complemented with rat *PDI* with strain YPH252 to form diploids. These diploids were cultured and sporulated, and the tetrads were dissected. The results obtained were similar to those seen with direct dissection of a disrupted cell line that had been transformed with the same plasmids. Only cells transformed with pMAL5.1 or pMAL9 were able to complement (data not shown). Spores that did not form colonies were examined by light microscopy. Approximately half of the nonviable spores did not germinate at all. The remainder proceeded through two or three cell divisions, and then died. We also dissected the tetrads at different times after the induction of sporulation, and found no change in viability. Thus, viability does not depend on the time spent sporulating, as

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had been observed in the complementation of a ubiquitin deficiency (Finley *et al.*, 1987).

Protein disulfide isomerase was isolated in 1964 on the basis of its ability to catalyze the isomerization of disulfide bonds (Givol *et al.*, 1964). Since then, it has been shown to be the β subunit of the MTP (an αβ dimer) (Wetterau *et al.*, 1990), the β subunit of prolyl 4-hydroxylase (an $\alpha_2\beta_2$ tetramer) (Pihlajaniemi *et al.*, 1987), and a thyroid hormone binding protein (Cheng *et al.*, 1987; Yamauchi *et al.*, 1987). This multiplicity of roles provokes the question of what cellular process or processes are impaired by a lack of PDI. At least one of these processes must be essential to *S. cerevisiae* cells because disruption of the *PDI1* gene is lethal to these cells (Farquhar *et al.*, 1991; LaMantia *et al.*, 1991; Scherens *et al.*, 1991; Tachikawa *et al.*, 1991).

The role of PDI in MTP function is unknown (Gordon *et al.*, 1995). Dimers formed between the α subunit and a mutated PDI subunit show that the enzymatic activity of the PDI subunit is not necessary for dimer assembly or MTP activity (Lamberg *et al.*, 1996). Still, PDI may play a direct role in the lipid transfer activity of MTP (Wetterau *et al.*, 1991). At a minimum, PDI appears to be necessary to maintain the structural integrity of MTP, and may enable MTP to remain in the ER by virtue of its C-terminal KDEL motif. Regardless, the absence of an MTP system in yeast as well as the viability of humans that lack MTP suggest that an MTP system, including its PDI component, is not essential for the life of eukaryotic organisms (Wetterau *et al.*, 1992).

PDI appears to be required to keep prolyl 4-hydroxylase in a catalytically active, non-aggregated conformation within the lumen of the ER. But as with MTP, the PDI subunit is not necessary for prolyl 4-hydroxylase assembly or enzymatic activity (Vuori *et al.*, 1992b). In addition, prolyl 4-hydroxylase that lacks PDI subunits has been found in species of green algae (Kaska *et al.*, 1988, 1990). Thus, the PDI subunits of prolyl 4-hydroxylase are not essential for eukaryotic cell viability.

Saccharomyces cerevisiae is an optimal system to examine the role of PDI *in vivo* because *S. cerevisiae* cells need PDI to live (Scherens *et al.*, 1991). We have developed a system that allows for both *in vivo* and *in vitro* studies of PDI (Laboissière *et al.*, 1995a). Here, we have used this system to determine the importance of ER import and retention of PDI in spore germination and cell division.

Rat PDI fused to the α-factor pre-pro segment and either the mammalian retention sequence KDEL or the yeast retention sequence HDEL enabled *PDI*-deficient cells to divide (Table 3). On the other hand, if PDI was fused to the α-factor pre sequence (a truncated form of the α-factor pre-pro segment that does not direct the protein to the external medium), doubling times were longer, indicating that rescue was less efficient (Table 4). Immunoblots demonstrated that little PDI is targetted to the ER by the α-factor pre sequence (Figure 2).

Rat PDI fused to the α-factor pre-pro segment and the yeast retention sequence HDEL enabled *PDI*-deficient spores to germinate (Table 2), albeit slowly (Figure 1). No other PDI construct was able to support germination. Further, half of the spores not rescued by PDI failed to germinate and the remainder proceeded through only a few cell divisions before ceasing growth. This phenotype, which had been found in similar experiments with other yeast genes (Naumovski and Friedberg, 1983; Mann *et al.*, 1987; Rose and Fink, 1987; Haggren and Kolodrubetz, 1988), suggests that nascent cells have a dire need for PDI. In a similar experiment, the germination of *pdi1*Δ spores containing a truncated PDI was observed to be inefficient (LaMantia *et al.*, 1991).

Our results indicate that passage of low levels of PDI through the ER is sufficient for vegetative cell division. In contrast, spore germination requires the efficient retention (via the HDEL motif) of large amounts (from the α-factor pre-pro segment) of PDI in the ER. These results are consistent with previous work by Kuntzel and coworkers, who showed that deletion of the 38 C-terminal residues of yeast PDI (including HDEL) is lethal if protein expression is directed by the ADC1 promoter (Günther *et al.*, 1991), but not if it is directed by the stronger GAL1 promoter (Günther *et al.*, 1993). Tetrad analysis does not allow as many cells to be observed as do some other methods. Nonetheless, the striking difference in the observed ability of rat *PDI* and yeast *PDI1* to complement during tetrad analysis argues against population size as being responsible for our results. In addition, the backcrosses rule out the possibility that during plasmid shuffling we selected for the over expression of *EUG1* or any other endogenous gene that would be able to rescue a PDI deficiency.

The essential role of PDI is to catalyze the unscrambling of nonnative disulfide bonds in ER proteins (Laboissière *et al.*, 1995b). In other words, PDI acts like an editor, correcting mistakes during protein folding in the cell. The results presented here show that this editorial activity is of greater consequence to germinating spores than to dividing cells.

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Short Communication

Cleavage of the Complement System C3 Component by HIV-1 Proteinase

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The C3 factor of the complement system and its C3b fragment are cleaved *in vitro* by the proteinase of the human immunodeficiency virus, type 1 (HIV PR). The cleavage occurs in the alpha-chain of both substrates at multiple sites yielding a 100 kDa fragment of the C3 alpha-chain and multiple fragments of the C3b alpha-chain. The scissile bonds are: Ala86-Glu87, Leu310-Leu311, His641-Trp642 and Arg649-Ser650. The resulting fragments resemble the physiologically occurring inactive fragments of C3: C3c and C3d, suggesting a possible biological role of the HIV-proteinase in the complement inactivation process.

Key words: Complement C3, C3b inactivation / Complement system / HIV proteinase.

The human complement system is efficiently activated by cell-free human immunodeficiency virus, type 1 (HIV), or HIV-infected cells, but neither HIV nor HIV-infected cells are lysed by the complement system (Banapour *et al.*, 1986, Marschang *et al.*, 1994). A virally-activated complement cascade generally proceeds up to the activation of C3, a central component of the complement system. This component consists of two polypeptide chains connected by disulfide bridges (Figure 1): the alpha-chain of 117 kDa and the β-chain of 75 kDa. In the process of activation, the complement-specific serine proteinase, C3-convertase, cleaves off the first 77 N-terminal amino acid residues from the alpha-chain resulting in the release of the small 10 kDa C3a fragment (Figure 1) and a subsequent conformational rearrangement of the remaining C3b molecule; this binds covalently to the target membrane via the thiol ester site (Müller-Eberhard, 1988). The deposition of C3b on the surface of HIV or infected cells has been demonstrated (Sölder *et al.*, 1989; Ebenbichler *et al.*, 1991).

A frequent process, however, used by viruses to counteract the activated complement cascade is a proteolytic or other modification of the C3b fragment bound to the target membrane. This mechanism is used by herpes viruses (HSV-1) (Friedman *et al.*, 1984), vaccinia virus (Kotwal and Moss, 1988) or EBV (Mold *et al.*, 1988) which, similar to HIV, activate the complement system, but are not lysed by it (Smith, 1994).

Blood of HIV-infected patients contains an enhanced concentration of inactive C3d and C3c fragments derived by proteolysis from active C3b fragments (Senaldi *et al.*, 1989); likewise, *in vitro* incubation of purified HIV with fresh normal human serum results in generation of fragments similar to C3d and C3c (Sölder *et al.*, 1989).

The principal task of the HIV-encoded aspartic proteinase (HIV PR) is to process viral gag and gag-pol polyprotein precursors to functional proteins thereby converting immature virions to infectious viruses. It has been reported that the HIV proteinase (PR) is also capable of cleaving *in vitro* a number of host cell proteins, like those of the cytoskeleton such as actin, actinin, vimentin, tropomyosin (Shoeman *et al.*, 1991; Tomasselli *et al.*, 1991; Tomasselli and Heinrikson, 1994), microtubule-associated protein, MAP (Wallin, 1990), Bcl-2 (Strack *et al.*, 1996) and extracellular matrix as well as blood plasma proteins (Oswald and von der Helm, 1991; Kisselev and von der Helm, 1994). The *in vivo* cleavage of vimentin, myosin (Lindhofer *et al.*, 1993) and actin (Adams *et al.*, 1992) could be demonstrated in HIV-infected cell culture to be due, in fact, to the proteolytic action of the HIV PR.

The occurrence of antibodies to HIV PR in the blood of more than one-third of HIV-infected patients (Boucher *et*

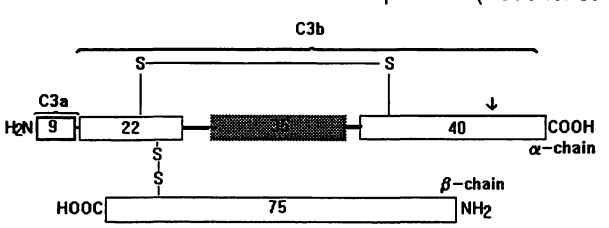

Fig. 1 Schematic Representation of the Human C3 Molecule. Boxes show the known proteolytic fragments of the molecule (Müller-Eberhard, 1988), among them C3d (shaded) and the fragments comprising C3c (not shaded). Thick lanes indicate the regions of the molecule sensitive to proteolysis (Clare O'Keefe, 1988; Müller-Eberhard, 1988). The numbers inside the boxes indicate molecular weights. The arrow indicates the HIV PR cleavage site in C3 (see text).

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al., 1989) suggests that active HIV PR might be present on the surface of infected lymphocytes (or viruses). Thus, it is conceivable that this proteinase attacks C3b molecules attached to the outside of cell membranes. These considerations prompted us to study whether the HIV-activated complement cascade, i.e. C3 or its C3b fragments, is sensitive to HIV PR cleavage.

We purified C3 from human plasma and incubated it with highly-purified recombinant HIV PR at pH 6.0 (most similar studies with HIV PR have been done at this pH). The reaction mixture was analyzed by SDS-PAGE under reducing conditions to detect possible cleavage fragments. In a control incubation without proteinase, both alpha- and beta-chains of C3 remained intact (Figure 2A, lane 1). Upon incubation with HIV PR at pH 6.0 the alpha-chain was partially cleaved, generating a 100 kDa fragment (lane 2). Microsequencing of this fragment revealed the known alpha-chain N-terminal sequence indicating that HIV PR cleaved close to the C-terminus (arrow on Figure 1). This cleavage could be blocked in a control experiment by addition of the specific peptidomimetic HIV PR inhibitor, Ro 31-8959 (Saquinavir), not shown.

C3b was experimentally obtained by treating purified C3 with trypsin (which is known to cleave C3 similarly to C3-convertase, as described by Pangburn, 1987) and subsequent purification on the Mono S column. When C3b was incubated with HIV PR the alpha-chain was cleaved at different sites generating six polypeptide fragments with molecular masses ranging from 30 to 45 kDa in SDS-PAGE under reducing conditions (Figure 2A, lane 4). No cleavage occurred in a control incubation of C3b without HIV proteinase (Figure 2A, lane 3). The N-terminal sequences of the HIV PR cleavage products determined by microsequencing revealed the following scissile bonds: Ala96-Glu87, Leu310-Leu311, His641-Trp642, Arg649-Ser650 (Table 1; amino acid numbering of native alpha-

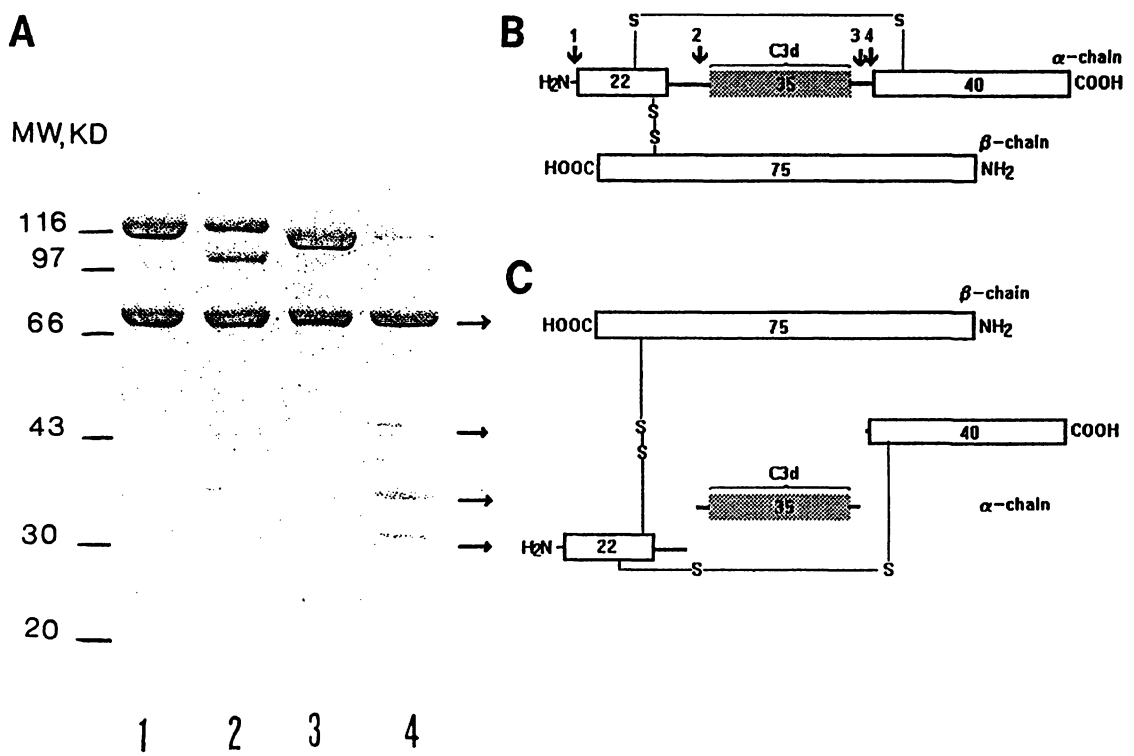

Fig. 2 Cleavage of C3 and C3b by HIV PR.

(A) SDS-PAGE of the reaction mixtures.

C3 and C3b were incubated at a concentration of 10 μM (1.8 mg/ml) for 17 hours at 37 °C with 0.5 μM HIV PR, or with HIV PR in the presence of its synthetic inhibitor Ro 31-8959 (Roberts *et al.*, 1990) at 2.5 μM, or without any proteinase. The reaction buffer was 20 mM MES, pH 6.0, 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.01% polyethyleneglycol 6000. The incubation mixtures were boiled in the gel-loading buffer and run on reducing SDS-PAGE (Laemmli, 1970).

Lane 1, C3, (incubation) without proteinase; lane 2, C3 with HIV PR; lane 3, C3b without proteinase; lane 4, C3b with HIV PR. Arrows at the bands point to the representing fragments (boxes) in Figure 2C.

Purification of substrates and enzyme. C3 was prepared from normal human plasma by a combination of PEG precipitation as well as DEAE-Sepharose FF (Hammer *et al.*, 1981), Phenyl-Sepharose (Janatova, 1988) and Mono S (Pangburn, 1987) column chromatography. C3b was generated from C3 by trypsin cleavage and purified on the Mono S column as described by Pangburn (1987). HIV PR was expressed in *Escherichia coli*, resolubilized and purified from insoluble material of the bacterial lysate as described (von der Helm *et al.*, 1994).

(B) Schematic representation of the C3b fragment with the same designations for C3 as in Figure 1. Arrows with numbers indicate the HIV PR cleavage sites; amino acid sequences at these sites are presented in Table 1.

(C) Schematic representation of HIV PR cleavage products using the same designations for C3 as in Figure 1. The alpha-chain fragments are located beside the bands in the SDS-PAGE (Figure A, lane 4), to which the arrows of Figure 2A refer.

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chain). These cleavages could also be blocked in a control experiment by the HIV PR inhibitor Saquinavir (data not shown). The half-life of C3b in the presence of HIV PR at a 1:20 molar ratio was approximately 1 h. Cleavage of C3b occurred 30 to 50-fold faster, than that of intact C3, and the cleavage pattern of C3b differed from that of C3, presumably because of the different conformation of these two proteins.

The elucidated scissile bonds of the C3b (Table 1) corroborated published data of HIV PR cleavage sites in other cellular proteins (Tomasselli and Heinrikson, 1994); this enzyme is able to cut peptide bonds not only between large hydrophobic residues (for example Leu*Leu) as are most of the aspartic proteinases, but also between charged and polar residues. Here we found Arg at P1 and Ser or Glu at P1′; at P2′ (bold in Table 1) we found Glu or Gln, which are highly preferred by HIV PR in this position (Dunn *et al.*, 1994, Tomasselli and Heinrikson, 1994).

All four peptide bonds in C3b cleaved by HIV PR (arrows 1, 2, 3, 4 in Figure 2B) are located very close to the cleavage sites used *in vivo* by other proteinases to convert C3b into inactivated fragments, such as C3d and C3c. The Ala86-Glu87 scissile bond (arrow 1) is only nine amino acid residues upstream of the C3 convertase site, generating C3a (and directly adjacent to the Glu87-Glu88 bond cleaved by the proteinase from cobra venom (Clare O'Keefe *et al.*, 1988)). The Leu310-Leu311 HIV PR scissile bond (arrow 2) is located between the factor I and trypsin site generating the N-terminus of the C3d. The His641-Trp642 bond (arrow 3) is in between the two factor I sites (Müller-Eberhard, 1988), and Arg649-Ser650 (arrow 4) coincides with the most upstream factor I scissile bond. Cleavage of the two Arg-Ser bonds by factor I is sufficient to inactivate C3b (Müller-Eberhard, 1988), suggesting that HIV PR cleavage might also lead to C3b inactivation.

The HIV PR produced a 35 kDa product (Figure 2A, lane 4) resembling very much the inactive C3d (Figure 2C) fragment [found in HIV-positive individuals (Senaldi *et al.*, 1989)]; it is slightly extended at both N- and C-terminus, i.e. fragment between arrows 2 and 3 or 4 in Figure 2B. The cleavage fragments of about 30 and that of about 43 kDa produced by the HIV PR (Figure 2A, lane 4) have similar masses as parts of the C3c fragments upon SDS-PAGE under reduced conditions (Clare O'Keefe *et al.*, 1988). According to their N-terminal sequences, these products are similar to 40 kDa and 22 kDa C3c fragments, respectively (open boxes in Figures 2B and 2C). Moreover, the cleavage product of 43 kDa (Figure 2A, lane 4) has the same size on SDS-PAGE as one of the fragments appearing after experimental incubation of HIV and human serum (Sölder *et al.*, 1989). The origin of the 38 kDa fragment is not clear (it was not subjected to microsequencing in our experiments).

The striking structural similarity of the fragments appearing after the *in vitro* reaction of C3b and HIV PR with those inactive C3d and C3c fragments found in human serum after HIV-infection favors a participation of the HIV PR in the impairment of the HIV-activated complement system. Taking into account that HIV infected individuals have a much higher load of virus particles than previously thought (Ho *et al.*, 1995; Wei *et al.*, 1995), it is conceivable that a similarly high ratio of HIV PR to C3b, as used in this study, may be reached on the surface of viruses or infected cells. Based on these results we suggest that the HIV PR might be involved in the impairment of HIV-activated complement by cleaving the C3b component.

Table 1 Amino Acid Sequences at the HIV PR Cleavage Sites in C3b.

No.	Amino acid sequence
1	Asp-Ile-Ile-**Ala*Glu**-Glu-Asn-Ile
2	Thr-Arg-Ile-**Leu*Leu**-Gln-Gly-Thr
3	His-Arg-Ile-**His*Trp**-Glu-Ser-Ala
4	Ser-Leu-Leu-**Arg*Ser**-Glu-Glu-Thr

The scissile bonds (*) were deduced by microsequencing of cleavage fragments (Figure 2A, lane 4) after blotting on Immobilon membranes. The number refers to the location of the cleaved bond in the molecule (see Figure 2B).

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