Poly(ADP-Ribose) Polymerase Is a Substrate Recognized by Two Metacaspases of *Podospora anserina*

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The two metacaspases MCA1 and MCA2 of the fungal aging model organism *Podospora anserina* (PaMCA1 and PaMCA2, respectively) have previously been demonstrated to be involved in the control of programmed cell death (PCD) and life span. In order to identify specific pathways and components which are controlled by the activity of these enzymes, we set out to characterize them further. Heterologous overexpression in *Escherichia coli* of the two metacaspase genes resulted in the production of proteins which aggregate and form inclusion bodies from which the active protein has been recovered via refolding in appropriate buffers. The renatured proteins are characterized by an arginine-specific activity and are active in caspase-like self-maturation leading to the generation of characteristic small protein fragments. Both activities are dependent on the presence of calcium. Incubation of the two metacaspases with recombinant poly(ADP-ribose) polymerase (PARP), a known substrate of mammalian caspases, led to the identification of PARP as a substrate of the two *P. anserina* proteases. Using double mutants in which *P. anserina* Parp (PaParp) is overexpressed and PaMca1 is either overexpressed or deleted, we provide evidence for *in vivo* degradation of PaPARP by PaMCA1. These results support the idea that the substrate profiles of caspases and metacaspases are at least partially overlapping. Moreover, they link PCD and DNA maintenance in the complex network of molecular pathways involved in aging and life span control.

Apoptosis is a type of programmed cell death (PCD) that is fundamental in removing unneeded cells from the body during development of multicellular organisms. In addition, in mammals, apoptosis plays a key role in removing severely damaged cells which are at risk of transforming into cancer cells. In unicellular and multicellular lower eukaryotes, like the yeast *Saccharomyces cerevisiae*, or in filamentous fungi, the role of PCD is less clear and leads to death of the whole organism or parts of it. Previously, it has been suggested that under natural conditions under which individuals compete for the available nutrients, PCD guarantees the conservation of the species via providing nutrients and other factors for survival of younger cells (1, 2).

Although differing in complexity, basic components and reactions associated with PCD are known to be conserved from lower eukaryotes to mammals. For instance, DNA fragmentation, the release of apoptotic factors from mitochondria, and the activation of specific cysteine proteases are hallmarks of apoptosis in all eukaryotic systems (3, 4). In fungi, plants, and protozoa, metacaspases were identified as sequence homologs of mammalian caspases (5). Caspases and metacaspases share structural similarities such as a common caspase hemoglobinase fold and conserved catalytic cysteine and histidine residues. Metacaspases can be subdivided into type I and type II analogs to mammalian initiator and effector caspases, respectively. The characteristic amino-terminal domain of type I metacaspases is reminiscent of CARD (caspase activation and recruitment domain) or DED (death effector domain) regulatory domains of initiator caspases although there is no sequence identity between these domains. In type II metacaspases and effector caspases, this kind of amino-terminal domain is lacking. Effector caspases contain only a short prodomain that was found to be involved also in the regulation of caspase maturation (6, 7). Also, the presence of a short prodomain in type II metacaspases cannot be excluded (8). A major difference between caspases and metacaspases exists in their substrate specificity. While metacaspases cleave their substrates after an arginine or lysine residue, caspases do so exclusively after aspartate (8–13).

Under physiological conditions, caspases and metacaspases control prosurvival processes. For instance, effector caspase-3 has been shown to be involved in the control of differentiation of different progenitor cells (14–16) and in the regulation of cell cycle events (17). Also, initiator caspases-8 and -9 play an important role in the development of different cell types (18, 19). In yeast, recently metacaspase YCA1 was demonstrated to be functional in the clearance of insoluble protein aggregates and also in controlling different checkpoints in cell cycle regulation (20, 21).

The PCD-related function of metacaspases is triggered by stimuli like acetic acid (22) or reactive oxygen species (ROS) (23–25). In particular, hydrogen peroxide appears to be a common inducer of metacaspases (26, 27) and of several caspases (28, 29). Significantly, this ROS and also metacaspase activity have been reported to increase during aging in different species (30–33), suggesting a role of metacaspases in the aging processes. This role is supported by genetic data. For instance, in yeast, chronological aging is delayed after deletion of the single metacaspase gene *Yca1* (1, 23, 25, 34). In the filamentous fungus *Podospora anserina*, the deletion of either of the two metacaspase genes leads to an increased life span compared to that of the wild type (32). Interestingly, the disruption of both metacaspases produced only a slight life span increase and a reduced growth rate and fertility, suggesting that the two metacaspases also have vital functions which can be expressed under natural conditions.
complement each other in the single-deletion strains (32). The direct involvement of metacaspases in PCD is further supported by the fact that *P. anserina Mca1* (*PaMca1*) deletion strains are more resistant against the common apoptosis inducer etoposide (31). Increased resistance against hydrogen peroxide of metacaspase deletion strains of *P. anserina* (32), *S. cerevisiae* (23), and *Candida albicans* (35) further supports the role of ROS as key regulators of metacaspase activity. In contrast, metacaspase deletion strains of *Aspergillus nidulans* showed no higher resistance to oxidative stress than wild-type strains but greater resistance to insults triggering endoplasmic reticulum (ER) stress, suggesting a role for metacaspases in developmental processes (36). In *Neurospora crassa*, programmed cell death occurred as a result of heterokaryon incompatibility accompanied by the generation of ROS. Since programmed cell death is executed metacaspase independently, these results suggest that an increase in ROS in general does not necessarily activate metacaspases in all fungal organisms (37).

To understand the role of metacaspases in more detail, it is important to know their biological substrates and thus the molecular pathways which are controlled by these enzymes. Currently, data about metacaspase substrates are rather limited. Recently, in *S. cerevisiae* and the Norwegian spruce *Picea abies*, the first specific substrates were identified (38, 39). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is cleaved in yeast by the type I metacaspase. The homologs are also cleaved by caspases (39, 44). Overall these limit the overlap. Specifically designed for overexpression of recombinant genes and the production of proteins (i.e., lack of LON and OMP1 proteases). Purification of protein from recombinant *E. coli* strains was performed using a RevertAid First Strand cDNA Synthesis Kit (47) and subsequently ground under liquid nitrogen for cell lysis. RNA isolation and cDNA synthesis. For heterologous production of *PaMca1*, *PaMca2*, and *PaParp*, we used *E. coli* strains which are specifically designed for overexpression of recombinant genes and the production of proteins (i.e., lack of LON and OMPIA proteases). Purification of protein from recombinant *E. coli* strains was performed by lysis of cells in buffer A (6 M guanidine hydrochloride, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 6.3). Elution of recombinant protein from Ni-NTA-agarose beads according to the instructions of the distributor (Qiagen, Hilden, Germany). Ni-NTA-agarose beads (Qiagen, Hilden, Germany) were added to extracts in order to bind the six-histidine-tagged recombinant protein to the beads. Subsequently, unbound nontagged proteins were separated from the solution by washing with buffer C (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 6.3). Elution of recombinant protein from Ni-NTA-agarose beads was prepared by a strong decrease in pH with buffer E (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 4.3) where nickel is not able to bind any histidine residues from the recombinant protein. Recombinant *PaMca1* (*rPaMca1*), *PaMca2* (*rPaMca2*), and *PaParp* (*rPaParp*) were purified as described in the QAexpression handbook (49) using Ni-NTA-agarose (Qiagen, Hilden, Germany).

Screening for refolding conditions. For refolding of recombinant *PaMCA1* and *PaPARP*, we used *E. coli* strains which are specifically designed for overexpression of recombinant genes and the production of proteins (i.e., lack of LON and OMPIA proteases). Purification of protein from recombinant *E. coli* strains was performed using overnight dialysis of cells in buffer A (6 M guanidine hydrochloride, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 6.3). Elution of recombinant protein from Ni-NTA-agarose beads was prepared by a strong decrease in pH with buffer E (8 M urea, 100 mM NaH2PO4, 10 mM Tris-HCl, pH 4.3) where nickel is not able to bind any histidine residues from the recombinant protein. Recombinant *PaMCA1* (*rPaMCA1*), *PaMCA2* (*rPaMCA2*), and *PaParp* (*rPaParp*) were purified as described in the QAexpression handbook (49) using Ni-NTA-agarose (Qiagen, Hilden, Germany).
Cloning procedure and generation of *P. anserina* mutants. For over-expression of PaMca1 in *P. anserina*, the open reading frame was amplified by PCR with oligonucleotides BamH1_McaIpre (5'-AAG GAT CCA TGT CTG GGT TTT TTC GGG C-3') and McaExrev (5'-CCA AGC TTA GCC ACC CTT TTT ACT CCT C-3'). Genomic DNA of *P. anserina* wild type was used as a template. To achieve constitutive expression, the PaMca1 DNA fragment was cloned into the expression vector pExMtpHph (52) downstream of the metallothionein promoter fragment. PaParp-overexpressing (OEx) strains and PaMca1 deletion strains were generated as described by Hamann et al. (32) and Müller-Ohldach et al. (48), respectively. Double mutant PaMca1(OEx) PaParp(OEx) and ΔPaMca1 PaParp (OEx) strains were generated by crossing two monokaryotic single mutants of the appropriate genotype. After generation of fruiting bodies and isolation of tetrads, ascospores carrying the two manipulated genes of interest were identified as double mutants and verified by Southern blotting.

Southern blot analysis. Southern blot analysis was performed as described previously (53). An hph gene-specific probe was constructed by digestion of vector pSM4 (54) with the restriction enzyme XhoI. A blt gene-specific probe was generated by digestion of vector pKO4 (55) with the restriction enzyme BamH1. The resulting 736-bp hph and 1,293-bp blt fragments were labeled with digoxigenin and used for genomic DNA hybridization of *P. anserina*.

Determination of metacaspase activity. Activity of recombinant metacaspases was detected by cleavage of the synthetic fluorescent substate urokinase III (Merck, Darmstadt, Germany) as previously described by Putt and Hergenrother (57). Therefore, a mixture of 5 μM recombinant PaParp (12.5 μg/ml) and nicked genomic DNA of the *P. anserina* wild type (75 μg/ml) was added to 20 μl of NAD\(^+\) (1.25 μM) in a 384-well plate to initiate the reaction following incubation for 20 min at room temperature. To determine the remaining amount of NAD\(^+\), the reaction mixture was treated with 10 μl of 2 M KOH and 10 μl of 20% acetylene for 10 min at 4°C. By the addition of 45 μl of 88% formic acid and incubation at 110°C for 5 min, a highly fluorescent compound was formed that was detected in a microplate reader (Sapphire 2; Tecan) with an excitation of 360 nm and an emission of 445 nm. NAD\(^+\) without PaParp was set to 100%. The procedure was performed in quadruplicate.

Preparation of nicked genomic DNA. Genomic DNA of the *P. anserina* wild type was isolated according to Lecellier and Silar (58). Referring to the protocol for preparation of PARP-activating DNA from The PARP Link (http://parplink.u-strasbg.fr), 30 μg of isolated genomic DNA of *P. anserina* was incubated with 2 μl of DNase I (Macherey-Nagel, Düren, Germany) for 60 s at room temperature. DNA was treated and precipitated as described in the protocol. Determination of damaged DNA was verified by 2% agarose gel electrophoresis.

rPaParp cleavage. Recombinant PaParp (2.5 μg) was incubated with 580 ng of either rPaMCA1 or rPaMCA2. When indicated (see Fig. 4C and D), 10 mM calcium chloride was added to the reaction mixture. Incubation was performed at 27°C in a range from 0 to 120 min. Samples were subsequently detected by Western blotting.

Nomenclature of substrate cleavage sites. According to the nomenclature described by Schechter and Berger (59), substrate amino acids are continuously numbered from the position they are cleaved by proteases. Upstream and downstream from the cleavage site, termed the scissile bond, amino acids are denoted as P1, P2, P3, etc. and as P1′, P2′, P3′, etc., respectively.

Schematic representation of *P. anserina* metacaspases and caspases. The picture and the representation of single subunits were generated according to protein annotations from UniProt and studies from yeast metacaspase YCA1 (13). The amino acid sequences for *P. anserina* metacaspases PaMCA1 and PaMCA2 are annotated in the *P. anserina* genome database (http://podospora.igmos.u-psud.fr). For demonstration and comparison of representative initiator and effector caspases, human caspase-8 and caspase-3 are mapped, respectively.

Statistical analysis. Statistical analyses between different samples were performed with Student’s *t* test. The minimum level for statistical significance was set as a *P* value of <0.05. In all analyses the means ± standard errors of the means (SEM) are shown.

RESULTS

Heterologous production and refolding of recombinant PaMCA1 and PaMCA2. For biochemical characterization of the two metacaspases PaMCA1 and PaMCA2, we set out to isolate the two proteins in purified and native forms. We used *E. coli* for heterologous production of recombinant proteins. In order to obtain a high yield of protein, the PaMca1 gene was codon optimized for *E. coli*, synthesized, and integrated into the expression vector pET21a + (Entelechon, Bad Abbach, Germany) (Fig. 1A). For the isolation of recombinant PaMCA2, we cloned PaMca2 in the expression vector pQE60 (Fig. 1B). In both constructs the metacaspase genes were fused to a six-histidine coding sequence at the expression vector pQE60 (Fig. 1B). In both constructs the metacaspase genes were fused to a six-histidine coding sequence at their 3′ ends, allowing the purification of the heterologous proteins via Ni-NTA-agarose. After transformation of *E. coli* with the corresponding hybrid plasmids, expression of the two metacaspases genes was induced with IPTG.

Since a number of attempts to generate soluble recombinant *P. anserina* metacaspases in *E. coli* were unsuccessful, we tried to resolubilize protein from putative aggregates. For resolubilization we selected commonly used, strong denaturants at high concentrations and purified 6 mg of recombinant PaMCA1 (rPaMCA1) and 4 mg of recombinant PaMCA2 (rPaMCA2) from 4 liters of *E.
coli cultures. Separation of 4 to 5 μg of purified recombinant protein from strains overexpressing PaMca1 and PaMca2 by SDS-PAGE and staining by Coomassie blue resulted in the identification of one major band at 54 kDa for both metacaspases. Using PaMCA1- and PaMCA2-specific polyclonal peptide antibodies, a 54-kDa and a 43-kDa protein detected the corresponding proteins as the full-length proteins and likely an incompletely processed form of the two metacaspases (Fig. 1C and D).

Since the purified proteins were unfolded in a nonphysiological buffer, renaturation in an appropriate buffer was necessary for subsequent biochemical characterization. To validate the success of an applied refolding procedure (for details, see Materials and Methods), E. coli protein from PaMca1-overexpressing strains was incubated in screening buffers with the lowest absorbance at 340 nm (buffers B1 to B5), indicative of dissolved protein. Screening buffers with the highest absorbance (B6 to B9) served as negative controls. After centrifugation of the samples, the supernatants were subsequently separated on an SDS gel following Coomassie staining (Fig. 1E). As expected, in the samples with the lowest absorbance, a protein migrating at about 54 kDa was detected. In addition, a low absorbance was measured for the water control (B2) loaded in lane 3 (Fig. 1E), suggesting a condition where rPaMCA1 becomes soluble. Samples with the highest absorbance contained no dissolved proteins. Most likely, in these buffers proteins precipitate and sediment during the centrifugation step and are lost from the supernatant fraction.

Next, we tested the protein extracts in the selected buffers for metacaspase activity. No cleavage of the synthetic metacaspase substrate (Gly–Gly–Arg–7-amino-4-methylcoumarin [AMC]) was observed. Therefore, we investigated whether dialysis of the proteins that although present in a nonaggregated form may not be correctly folded can lead to the native protein with metacaspase activity. After dialysis against the screened refolding buffers (Table 1, buffers B1, B3, B4, and B5) rPaMCA1 was active in cleaving the synthetic substrate Gly–Gly–Arg–AMC (Fig. 1F). The highest arginine-specific activity (Fig. 1F) was detected from rPaMCA1 after refolding in buffer B4. Treatment of rPaMCA2 according to the same procedure and using the same buffers also resulted in cleavage of the metacaspase-specific synthetic substrate.

Activity and autocatalytic processing of P. anserina metacaspases are calcium dependent. From in silico analyses of P. anserina metacaspases, we obtained the information that both enzymes possess an amino-terminal proline-rich extension characteristic for type I metacaspases (Fig. 2). Such metacaspases are structurally related to initiator caspases and can be divided into three segments: a prodomain, a p20 subunit, and a p10 subunit. In mammals, the maturation of initiator caspases depends on at least two cleavage events. First, cleavage takes place between
the prodomain and the p20 subunit. Subsequently, cleavage separates the p20 and the p10 subunits, and both subunits form a heterodimer and are attached to another heterodimer. Next, they are released from the prodomain, leading to the mature homodimeric enzyme (60). From baker’s yeast (13), Arabidopsis thaliana (11), and Allomyces arbuscula (61), it is known that maturation and activation of metacaspases, in contrast to caspses, is calcium dependent. Therefore, we tested the effect of different calcium concentrations on the proteolytic activity of rPaMCA1 and rPaMCA2. In the activity assay both P. anserina metacaspases exhibited calcium-dependent arginine-specific cleavage activity of the synthetic substrate. The activity increased with higher calcium levels and decreased after reaching an optimum (Fig. 3A and B). The decrease in activity was observed at calcium concentrations above 20 and 50 mM for rPaMCA1 (Fig. 3A) and rPaMCA2 (Fig. 3B), respectively, which can be explained by salt precipitation of both recombinant proteases as a result of excess calcium concentrations. Next, we analyzed auto-cleavage during calcium-mediated activation of the P. anserina metacaspases. In these assays we incubated either rPaMCA1 or rPaMCA2 with different amounts of calcium or with a 12.5 mM concentration of the calcium chelator EGTA to scavenge residual calcium ions and analyzed samples of both proteases by Western blot analysis using specific peptide antibodies (Fig. 3C and D). These analyses revealed a calcium-dependent auto-processing of both metacaspases. Several protein bands appeared in both analyses that can be attributed to incomplete metacaspase processing. Even low concentrations of 1 mM calcium spontaneously generated an additional 15-kDa fragment that is missing in calcium-depleted extracts (EGTA) and is associated with the increase in calcium-dependent protease activity. This low autocatalytic processing activity increases with increasing calcium concentrations, leading to a decrease in the full-length protein. Overall, this analysis revealed a calcium-dependent caspase-like auto-processing of both metacaspases from of P. anserina.

**TABLE 1 Absorption of refolding approach at 340 nm**

| Buffer name | Buffer composition<sup>a</sup> | pH | Absorption (340 nm) |
|-------------|--------------------------------|----|---------------------|
| B1          | HEPES, l-Arg, GSH+GSSG, NaCl+KCl | 7.5 | 0.0465             |
| B2          | ddH<sub>2</sub>O                  | 7.0 | 0.0704             |
| B3          | TAPS, l-Arg, metals, TCEP, NaCl+KCl | 8.5 | 0.0715             |
| B4          | MOPS, l-Arg, GSH+GSSG, NaCl+KCl  | 7.0 | 0.0792             |
| B5          | EPPS, l-Arg, TCEP, NaCl+KCl      | 8.0 | 0.1077             |
| B6          | EPPS, Tween 20, EDTA, GSH+GSSG   | 8.0 | 0.267              |
| B7          | MOPS, Tween 20, metals, TCEP      | 7.0 | 0.2947             |
| B8          | HEPES                             | 7.5 | 0.2969             |
| B9          | EPPS, Tween 20, metals, NaCl+KCl  | 8.0 | 0.3183             |

<sup>a</sup> Recombinant PaMCA1 was added to 48 different buffers, and absorption was measured at 340 nm. Samples with the five lowest and four highest absorptions and their corresponding buffer compositions are listed.

<sup>b</sup> GSH, glutathione; GSSG, glutathione disulfide; ddH<sub>2</sub>O, double-distilled H<sub>2</sub>O; TAPS, N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TCEP, Tris-(2-carboxyethyl) phosphine; EPPS, N-(2-hyroxyethyl)piperazine-N'- (3-propanesulfonic) acid.

**FIG 2** Comparison of the domain structures of P. anserina metacaspases and mammalian caspases. In P. anserina two metacaspases, PaMCA1 and PaMCA2, were identified as classical type I metacaspases with a proline-rich prodomain, which is reminiscent of the CARD (caspase activation and recruitment domain)- or DED (death effector domain)-containing domain of mammalian initiator caspases (e.g., human caspase-8). Mammalian effector caspases (e.g., human caspase-3) contain only a short prodomain without a recruitment domain which is involved in the regulation of caspase activity. Like all caspses, metacaspases also contain two subunits, termed p20 and p10, corresponding to their approximate protein sizes. Metacaspases and caspases share catalytic histidine and cysteine residues, which are indicated as white bars.
verify whether PaPARP folding was successful, we detected the rPaPARP activity with a nonradiometric assay by measuring the consumption of NAD$^+$ as previously described (57). This assay relies on the requirement of poly(ADP-ribose) polymerase activity on NAD$^+$ (48, 64). PaPARP activity is indicated by the consumption of NAD$^+$ in a fluorometric in vitro assay (57). Incubation of NAD$^+$, nicked genomic DNA of P. anserina wild type, and recombinant PaPARP led to a 20% reduction of NAD$^+$ (Fig. 4B), demonstrating that rPaPARP was successfully refolded and enzymatically active.

In vitro cleavage of rPaPARP by rPaMCA1 and rPaMCA2. In mammals, PARP-1 is a substrate of caspase-3 and -7 during apoptotic cell death and is cleaved at a single specific amino acid sequence (DEVD) after an aspartate residue at position P1, leading to the generation of 89- and 24-kDa fragments (13, 15). The latter fragment contains the active site and the DNA-binding domain. PARP loses the ability to recognize and bind to damaged DNA, a process that is essential for the activation of this protein.

To test whether PaPARP is a substrate of the P. anserina metacaspases and, if so, whether cleavage leads to a loss of function of the enzyme, we incubated recombinant PaPARP with each of the recombinant metacaspases PaMCA1 (Fig. 4C) and PaMCA2 (Fig. 4D). For metacaspase activation, 10 mM calcium was added to the reaction mixture. Incubation time was between 0 and 120 min. As a control, rPaPARP was incubated alone with and without calcium to ensure that rPaPARP is not degraded over time or by the addition of calcium. All samples were separated by SDS-PAGE and analyzed by Western blot analysis. The PaPARP-specific peptide antibody detected the full-length protein (77 kDa) and cleavage products (~56 kDa) of PaPARP that were already processed during heterologous production in E. coli. Incubation of rPaPARP and rPaMCA1 showed a time-dependent cleavage of rPaPARP (Fig. 4C). After an incubation time of 120 min, the abundance of full-length rPaPARP at 77 kDa was clearly reduced. The control sample including rPaPARP and rPaMCA1 without calcium resulted in no degradation of rPaPARP, demonstrating that metacaspase activation by calcium is crucial for rPaPARP cleavage. Interestingly, a cleavage product was detected by the PaPARP antibody at about 70 kDa and appeared after 15 min of incubation. Its intensity was slightly increased after 60 min, and it disappeared completely after 120 min of incubation.

Cleavage of rPaPARP by rPaMCA2 (Fig. 4D) differs from cleavage by rPaMCA1. Incubation of rPaPARP and rPaMCA2 for 120 min led to no apparent reduction in the abundance of full-length rPaPARP (77 kDa). The same cleavage product that was also detected in the experiment with rPaMCA1 at approximately 70 kDa was generated quickly after a few minutes and remained until the end of incubation. When rPaPARP and rPaMCA2 were incubated without calcium, no cleavage product appeared. This is in accordance with the results derived by the incubation of rPaPARP and rPaMCA1 showing that calcium is essential for rPaPARP cleavage by rPaMCA2.

In order to investigate the effects of metacaspase-mediated rPaPARP cleavage (Fig. 4C and D), we tested whether rPaPARP is still functional after incubation with rPaMCA1 or rPaMCA2. Prior to detection of PARP activity, rPaPARP was incubated either without or with rPaMCA1 or rPaMCA2 for 2 h at 27°C (Fig. 5). Enzymatic function of rPaPARP was measured by the consumption of NAD$^+$. To activate rPaPARP, nicked genomic DNA of P. anserina was added to the reaction mixture. The results show that,
form, confirmed by the consumption of NAD\(^+\) two overnight steps at 4°C, the protein was obtained in a refolded and active antibody. (B) After dialysis against buffer B4 and metacaspase assay buffer in Coomassie staining and immunodetection with a PaPARP-specific peptide expected size of rPaPARP (77 kDa) was confirmed by SDS-PAGE following of recombinant PARP which was purified under denatured conditions. The serinal metacaspases. (A) Heterologous production of rPaPARP yielded 2.7 mg PARP activity itself.

**DISCUSSION**

In the current study, we report the successful heterologous production of two metacaspases and of poly(ADP-ribose) polymerase from *P. anserina* in *E. coli*. All proteins formed inclusion bodies similar to results with the NAD\(^+\) control without rPaPARP, NAD\(^+\) levels are not reduced after incubation of rPaPARP with rPaMCA1, indicating inactivation of rPaPARP by rPaMCA1. In contrast, incubation of rPaPARP with rPaMCA2 has no impact on the NAD\(^+\) consumption of rPaPARP. NAD\(^+\) consumption is the same as measured with rPaPARP alone. In this assay, cleavage of rPaPARP by rPaMCA2 is not sufficient to reduce the enzymatic activity of recombinant poly(ADP-ribose) polymerase, suggesting that generation of the 70-kDa intermediate has no effect on the PARP activity itself.

**In vivo degradation of PaPARP in *P. anserina***. Next, we aimed to demonstrate that PaPARP is also an *in vivo* substrate of PaMCA1. Since we know from earlier experiments that PaPARP can be detected only by the PaPARP-specific peptide antibody in strains which overexpress *Parp* (48), we used such overexpressors in subsequent experiments. After crossing a PaMca1-overexpressing strain with a *PaMca1*-overexpressing strain or a *PaMca1* deletion strain, we isolated two double mutants. One was a strain overexpressing *PaMca1* and *PaParp* and the other strain was a *PaParp*-overexpressing strain in which the *PaMca1* gene was deleted. The double mutants were verified by Southern blotting and by measuring arginine-specific activity (Fig. 6). While basically no difference was observed in extracts from mycelia of both strains grown on synthetic medium without hydrogen peroxide, overall arginine-specific protease activity (32) was increased in both mutants grown on medium supplemented with hydrogen peroxide (Fig. 6C). Moreover, *PaMca1(OEx) PaParp(OEx)* mutants are characterized by a significant increase in protease activity compared to *PaMca1 PaParp* (OEx) mutants (Fig. 6C). These data are consistent with earlier data which revealed an induction of metacaspase activity by hydrogen peroxide (32).

Next, we investigated protein extracts of the different strains for PaPARP abundance. In agreement with earlier data, no PaPARP was observed in the wild type, the *PaMca1*-overexpressing strain, or the deletion strain. The full-length PaPARP protein of 75 kDa and a number of degradation products were identified only in the *PaParp*-overexpressing strain (Fig. 7A). Importantly, when grown on hydrogen peroxide-containing medium, all four independent *PaParp*-overexpressing strains (four biological replicates) lacking PaMCA1 showed a significant 1.8-fold increase in the amount of the 75-kDa PaPARP protein compared to the four biological replicates of the deletion strain. The full-length PaPARP protein of 75 kDa observed in the wild type, the *PaMca1*-overexpressing strain, or the deletion strain was consistently reduced in the *PaMca1* deletion strain. The double mutants were verified by Southern blotting and by measuring arginine-specific activity (Fig. 6).

FIG 4 Cleavage of recombinant refolded and active PaPARP by both *P. anserina* metacaspases. (A) Heterologous production of rPaPARP yielded 2.7 mg of recombinant PARP which was purified under denatured conditions. The expected size of rPaPARP (77 kDa) was confirmed by SDS-PAGE following Coomassie staining and immunodetection with a PaPARP-specific peptide antibody. (B) After dialysis against buffer B4 and metacaspase assay buffer in two overnight steps at 4°C, the protein was obtained in a refolded and active form, confirmed by the consumption of NAD\(^+\) in the PARP activity assay. The control contained NAD\(^+\) and nicked DNA. ***, \(P = 6 \times 10^{-4}\) (Student's *t* test). (C) Incubation of rPaPARP with rPaMCA1 resulted in visible degradation of full-length rPaPARP after 120 min as detected by the PaPARP-specific peptide antibody. A metacaspase-generated 70-kDa intermediate that was present until 60 min of incubation disappeared together with intermediates at 54 kDa. The reaction mixture of rPaPARP and rPaMCA1 without calcium showed no rPaPARP cleavage. (D) Cleavage after incubation of rPaPARP and rPaMCA2 is different from that of rPaMCA1. The 70-kDa intermediate stabilizes during an incubation time of 120 min. Degradation of full-length rPaPARP was not detected by the antibody.

FIG 5 rPaPARP activity assay after incubation with metacaspases. Prior to detection, rPaPARP was either incubated with recombinant PaMCA1 or PaMCA2 or left untreated. After that, consumption of NAD\(^+\) was measured by the PARP activity assay. When rPaPARP is incubated with rPaMCA1, there is no consumption of NAD\(^+\) by rPaPARP. Incubation of rPaPARP and rPaMCA2 resulted in no change in NAD\(^+\) consumption compared to that of untreated rPaPARP. The control contained NAD\(^+\) and nicked DNA without rPaPARP. Significance (***)) was determined as follows: control versus rPaPARP, \(P = 1 \times 10^{-5}\); control versus rPaPARP incubated with rPaMCA2, \(P = 1 \times 10^{-6}\) (Student’s *t* test).
and had to be solubilized and refolded to generate an active protein for biochemical characterization. Three of four buffers selected for refolding had similar compositions. All buffers contained L-arginine, which seems to be essential for correct refolding of the *P. anserina* proteins. Previous studies investigating the activity of metacaspases revealed that *in vivo* the induction of these proteases is triggered after hydrogen peroxide treatment (26, 27). In *P. anserina*, metacaspase–specific peptidase activity was described in senescent but not in juvenile mycelia from the wild type (32). Since deletion of *PaMca1* resulted in increased resistance against hydrogen peroxide, this ROS is likely to be an inducer of metacaspase activity also in *P. anserina* (32). In the yeast *Candida albicans* and the protozoan *Leishmania donovani*, hydrogen peroxide treatment was found to lead to an increase in cytosolic calcium levels and aspartate-specific cleavage activity (65, 66). In *S. cerevisiae, Arabidopsis thaliana*, and other organisms, a calcium-dependent arginine-specific *in vitro* activity, which is characteristic for metacaspases, was reported (11, 61, 67). The same dependency on calcium was found in our study. Western blot analyses with specific peptide antibodies against each metacaspase revealed that even low concentrations of calcium processed both metacaspases into smaller fragments to become the mature active enzyme. Most probably, this is mediated by autocatalytic cleavage. The synthetic substrate used in our *in vitro* activity assay was processed considerably faster by rPaMCA2 than by rPaMCA1. This difference suggests that there is a preference in substrate recognition, probably dependent on the amino acid sequence.

It is known that mammalian initiator caspases become activated by recruiting their precursors (procaspases) to protein complexes, like the death-inducing signaling complex (DISC) for caspase-8, leading to an increase in local procaspase concentration and autocalytic cleavage, a process termed “proximity-induced activation” (68). Activation of effector caspases is subsequently mediated by cleavage of upstream (initiator) caspases (69). Therefore, the generation of a small fragment is indicative of maturation and activation of this class of enzymes. The small metacaspase fragment with approximately 15 kDa detected for rPaMCA1 and rPaMCA2 (Fig. 3C and D) corresponds to the p10 subunit of...
caspases that is separated from the p20 subunit during activation. In contrast to metacaspases, there is no direct calcium-dependent processing known for caspases (69). In S. cerevisiae, a short form of metacaspase YCA1 was biochemically characterized and found to result from autocatalytical cleavage at specific amino acids (13). In contrast, the analyses in this study were performed with full-length P. anserina protein showing several unidentified fragments even in the presence of the specific calcium chelator EGTA. Probably both metacaspases are already processed at certain sites when they become refolded in the native buffer that does not lead to fully activated enzymes. For yeast metacaspase YCA1, several auto-processing sites were identified. Arg72/Lys86 and Lys331/Lys334 are probably responsible for the release of the prodomain and the cleavage between the p20 and p10 subunits, respectively. Corresponding to these auto-processing sites of YCA1, putative cleavage sites were also found in P. anserina metacaspases which could be responsible for separation of these three domains.

For separation of the PaMCA1-specific prodomain from the p20/p10 subunits, 10 amino acid residues in the peptide sequence of the protease were identified as putative auto-cleavage sites. However, only one cleavage site at Arg86 is likely to be responsible for the protein identified in Western blotting below the 43-kDa marker band, which appears to represent the single p20/p10 fragment (Fig. 3C). An auto-cleavage event separating the p20 and p10 subunits to become the mature enzyme may occur at amino acids Lys293 and Lys299 of PaMCA1. Two Western blot signals below the size of 17 kDa may represent the p10 subunit that is differentially processed at both lysine sites. This conclusion is supported by
studies with yeast metacaspase YCA1 (13) and Trypanosoma brucei metacaspase MCA2 (TbMCA2) (70). In the latter study it was shown that a hydrophobic amino acid is located in the P2 position, upstream of the lysine in P1 position.

For PaMCA2, Arg120 and Arg125 are good candidates for cleavage sites for separation of the prodomain from the p20/p10 subunits. In our Western blot analysis the corresponding protein may be the double band of a size below 43 kDa (Fig. 3D). The protein migrating at about 26 kDa can be explained by additional cleavage sites at Lys193 or Arg194 resulting also in a smaller p20/p10 fragment separated from the prodomain. As shown for both lysine cleavage sites of PaMCA1, there is also a hydrophobic amino acid in the P2 position next to Lys193 of PaMCA2. An auto-cleavage event that separates the p20 from the p10 subunit to become the mature enzyme is likely to occur at amino acids Lys351 and Lys355. This conclusion is supported by the two Western blot signals below the 17-kDa marker band representing the p10 subunit.

Using the recombinant and active P. anserina metacaspases, we show that recombinant PaPARP, the homolog of mammalian PARP-1, is cleaved by both P. anserina metacaspases in vitro. Mammalian PARP is known to be a multifunctional enzyme involved in DNA repair, regulation of chromatin structure, transcriptional regulation, mitosis, telomere maintenance, and cell death pathways (64) and is essential for controlling cell homeostasis. Its primary function is to detect and repair DNA damage in an NAD$^+$-consuming process (71). Cleavage of mammalian PARP-1 is a hallmark of apoptosis. During apoptosis the enzyme is cleaved by caspase-3 and -7 (71), leading to the generation of 89- and 24-kDa fragments. The large fragment containing the catalytic site translocates to the cytosol while the small fragment with the DNA binding domain is retained in the nucleus, where it can bind to damaged DNA that is no longer recognized by intact PARP-1 to initiate its repair activity.

Interestingly, recombinant PARP of P. anserina is differentially processed by PaMCA1 and PaMCA2. The efficiency of cleavage by rPaMCA1 appears to be higher than that by rPaMCA2. The 70-kDa cleavage product generated after a short time of incubation as well as rPaPARP intermediates of approximately 55 kDa are degraded completely after 2 h of incubation. This progressive cleavage of rPaPARP may be explained by the presence of 25 arginine and 62 lysine residues representing potential cleavage sites of metacaspases. It is conceivable that there is a preference of metacaspases recognizing specific amino acids upstream of the P1 position of their substrate as it is characteristic for mammalian caspases (72). The amino acid sequences DEVD, YVAD, or VAD are known to be common cleavage sites for aspartate-specific caspases (8) but not for arginine- and lysine-specific metacaspases. It appears that rPaPARP loses activity as a result of rPaMCA1-mediated cleavage. In contrast, cleavage by PaMCA2 does not affect NAD$^+$ consumption, indicating that PaPARP activity of the cleaved enzyme is not impaired. It appears that a 70-kDa rPaPARP intermediate generated by cleavage retains the active site for enzymatic function consuming NAD$^+$ (Fig. 5).

A recent study with the T. brucei metacaspase TbMCA2, which is highly conserved like PaMCA1 and PaMCA2, revealed a cleavage preference for specific amino acids upstream and downstream of the P1 position of the substrate (73). While basic amino acids in the P2 and P3 positions promote substrate cleavage, cleavage is inhibited by negatively charged amino acids in position P2 or P3 or aromatic amino acids in position P1’. These criteria seem to apply, at least in part, also for A. thaliana metacaspase MC9 (AtMC9) (74). Taking these parameters into account, several putative cleavage sites can be found in the PARP of P. anserina. Specifically, these are 4 arginine- and 11 lysine-specific amino acid sequences (Fig. 8). Since catalytic activity of rPaPARP is unaffected after intermediate generation by PaMCA2, this supports the idea that at least one cleavage must occur in the amino-terminal region of the PaPARP enzyme and not in the carboxy terminally located catalytic PARP domain. Three of the 15 putative cleavage sites could explain the generation of PaPARP intermediates between 63 and 73 kDa, leaving the PARP domain unaffected. Hydrolysis of the PaPARP protein at these sites would affect only the BRCT domain that was described as an auto-modification domain (48). The putative DNA-binding domain WGR and also the catalytic PARP domain are not affected by the proposed cleavage events.

Until now, in lower eukaryotes, a correlation between caspase-like activity and PARP degradation has been reported in only a few cases. For example, in A. nidulans it was shown that PARP is cleaved during sporulation (75). In the protozoan L. donovani, PARP degradation in correlation with an increase in caspase-like activity was observed during hydrogen peroxide-induced apoptosis (66). However, since in both studies aspartate-containing substrates were used in the activity assays and not metacaspase-spe-
specific arginine or lysine substrates, a metacaspase-mediated cleavage of PARP in these organisms is not yet proven.

In addition to the demonstration of PaPARP as an in vitro substrate of recombinant PaMCA1, we here provide the first evidence that this metacaspase also cleaves PaPARP in vivo. In strains both overexpressing PaParp and lacking PaMCA1, significantly more of the full-length PaPARP was found in total protein extracts of strains grown on hydrogen peroxide-containing medium than in strains overexpressing both PaParp and PaMCA1. PaMCA1 thus appears to control PARP abundance in response to oxidative stress.

A key biological function of PARP in the control of DNA maintenance and aging of biological systems has been elucidated in previous studies. In a pioneer study the enzyme activity of PARP in mononuclear leukocytes from 13 animal species was linked with the different maximum life spans of these organisms (76).

Cells from species with a longer maximum life span (e.g., humans or gorillas) showed higher PARP activity than those of shorter-lived species (e.g., rats and rabbits). In this study, it was also shown that it is the activity rather than the abundance of PARP in the cells that correlated with life span. Since aging is associated with genetic instability triggered by inducers of DNA damage like ROS or other endogenous and exogenous insults, it was concluded that a higher PARP activity antagonizes the accumulation of DNA damage more efficiently in longer-lived organisms (76). In our studies with the P. anserina wild type, which is characterized by a very short life span of about 25 days, we were not able to detect the protein in Western blot experiments (Fig. 7), nor could we measure PARP activity in wild-type extracts. Only in PaParp-overexpressing strains was this possible (48). These results suggest a rather low abundance of functional PaPARP in this short-lived organism. As suggested by the current study, functional PaPARP may even become reduced by PaMCA1 cleavage. Overall, these data suggest that a restricted DNA maintenance system is a bottleneck in the cellular quality control network. Our earlier attempts to strengthen this branch of the system by overexpression of PaParp failed (48), most likely due to the fact that, in such transgenic strains, NAD$^+$ levels become depleted, leading to functional impairments and reduced life span. Efficient interference in age-related pathways related to DNA maintenance, PARP function, and apoptotic pathways requires a detailed understanding of the various components active in the corresponding pathways, including their regulation and interactions. Unfortunately, even in lower systems, in which, for instance, pathways controlling PCD are much simpler than in higher systems, we are far from such an understanding. For instance, we do not know how metacaspases become activated by calcium after treatment with hydrogen peroxide. Also, the precise mechanisms leading to the subsequent cleavage of biological substrates like PARP remain to be unraveled.

At this time, we propose the following scenario to take place during aging of P. anserina. During respiration the primary ROS superoxide is progressively generated at the mitochondrial respiratory chain. This ROS is converted to hydrogen peroxide via the activity of mitochondrial superoxide dismutase and is able to cross membranes. After accumulation of hydrogen peroxide in the cytoplasm, calcium is released from intracellular stores like the endoplasmic reticulum (ER) or mitochondria, promoting the maturation of metacaspase precursors, which subsequently become activated. Once active, metacaspases cleave cellular substrates including PaPARP and also other, yet unknown, essential proteins, leading eventually to the death of the whole individual.

Overall, in our study we reported the successful development of a procedure to generate active proteins after in vivo expression of the corresponding genes in E. coli. The proteins are accessible for biochemical analyses. In such analyses we identified the poly-(ADP-ribose) polymerase as the first specific substrate of P. anserina metacaspases in vitro and raised evidence for degradation of PaPARP by PaMCA1 in vivo in response to oxidative stress. Although caspases and metacaspases are characterized by different cleavage properties, our data support the idea that they may have at least a partially overlapping substrate profile. The identification of these substrates and their precise characterization hold a key to unraveling yet unknown pathways involved in the complex network controlling development and biological aging.

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