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Research in Microbiology 156 (2005) 35–46
Research in Microbiology
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Proper folding of the antifungal protein PAF is required for optimal activity

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Received 30 April 2004; accepted 13 July 2004

Available online 11 August 2004

Abstract

The *Penicillium chrysogenum* antifungal protein PAF is secreted into the supernatant after elimination of a preprosequence. PAF is actively internalized into the hyphae of sensitive molds and provokes growth retardation as well as changes in morphology. Thus far, no information is available on the exact mode of action of PAF, nor on the function of its prosequence in protein activity. Therefore, we sought to investigate the effects of secreted PAF as well as of intracellularly retained pro-PAF and mature PAF on the sensitive ascomycete *Aspergillus nidulans*, and transformed this model organism by expression vectors containing 5′-sequentially truncated *paf*-coding sequences under the control of the inducible *P. chrysogenum*-derived xylanase promoter. Indirect immunofluorescence staining revealed the localization of recombinant PAF predominantly in the hyphal tips of the transformant Xylpaf1 which expressed prepro-PAF, whereas the protein was found to be distributed intracellularly within all segments of hyphae of the transformants Xylpaf2 and Xylpaf3 which expressed pro-PAF and mature PAF, respectively. Growth retardation of Xylpaf1 and Xylpaf3 hyphae was detected by proliferation assays and by light microscopy analysis. Using transmission electron microscopy of ultrathin hyphal sections a marked alteration of the mitochondrial ultrastructure in Xylpaf1 was observed and an elevated amount of carbonylated proteins pointed to severe oxidative stress in this strain. The effects induced by secreted recombinant PAF resembled those evoked by native PAF. The results give evidence that properly folded PAF is a prerequisite for its activity.

Keywords: Antifungal protein PAF; *Penicillium chrysogenum*; *Aspergillus nidulans*; Prepro-protein; Heterologous gene expression; Growth retardation; Oxidative stress

1. Introduction

The antifungal protein PAF is a small, basic cysteine-rich protein secreted by the β-lactam-producing filamentous fungus *Penicillium chrysogenum* Q176 [17]. PAF inhibits the growth of various filamentous fungi, among others plant pathogenic and opportunistic zoopathogenic molds. The effects of PAF on sensitive organisms were characterized as being multifactorial, including induction of a crippled phenotype, metabolic inactivation, membrane permeabilization and oxidative stress [13]. Recently, indirect immunofluorescence studies revealed that PAF is actively transported into the cells of sensitive fungi, which indicates that the detrimental effects are provoked from inside the cells [20].

PAF is synthesized as a precursor protein with a signal sequence of 18 amino acid (aa) residues and a prosequence of 19 aa which is located N-terminally to the mature protein (55 aa) [17]. Apart from the signal peptide for secretion via the endoplasmic reticulum, many extracellular proteins of pro- and eukaryotes also possess proregions at the N-terminus, C-terminus or even within the primary

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0923-2508/$ – see front matter
doi:10.1016/j.resmic.2004.07.007

sequence, which are cleaved off during maturation of the precursor protein. In many cases, mature proteins gain their activity upon removal of these prosequences, e.g., in the case of zymogens, growth factors, neuropeptides, prohormones and toxins [19]. An additional function of the prosequence is to act as an intramolecular chaperone by enabling proper folding of the mature protein portion [12]. In contrast to molecular chaperones, intramolecular chaperones are covalently linked to the protein to be folded.

Our special interest in this study was to investigate the effect of the heterologous expression of recombinant PAF in the sensitive mold *Aspergillus nidulans* in order to gain better insight into the relationship of the primary structure to the function of the antifungal protein. Efficient expression of recombinant proteins by induction with xylan or xylose via the *P. chrysogenum*-derived xylanase (*xylP*) promoter has been shown in the model organism *A. nidulans* [34]. Using this expression system, we characterized PAF-sensitive *A. nidulans* strains which expressed the recombinant, secreted prepro-PAF, and the intracellularly retained pro-PAF and mature PAF upon induction. The effects of expression of the different recombinant PAF types on the producing transformants were analyzed.

2. Materials and methods

2.1. Strains, media, growth conditions and activity assays

Vectors and plasmids were propagated in *Escherichia coli* DH5α (DSM 6897). The fungal strains used in this study were *A. nidulans* WG355 (*biA1 bgaO argB2*), *A. nidulans* wt (FGSC A4), *A. niger* (CBS120.49) and *P. chrysogenum* Q176 (ATTC10002). For transformation, *A. nidulans* was grown in minimal medium MM according to Pontecorvo (1953) [22] at 37 °C with glucose as carbon source. Transformants were cultivated in complete medium CM [13] or MM, containing 2% glucose for repressing and 1% xylose for inducing growth conditions at 30 °C. Media were supplemented as required. *P. chrysogenum* was grown in *Penicillium* minimal medium at 25 °C and PAF was purified from the supernatant as described previously [17]. The growth of *paf*-expressing *A. nidulans* strains was determined by spotting 5×10^3 conidia in 5 µl aliquots on solid medium. Alternatively, 200 µl liquid CM were inoculated with 1×10^4 conidia/ml in 96-well microtiter plates and growth was determined spectrophotometrically as described [6,13,15].

2.2. Construction of plasmids

For cloning procedures, standard recombinant DNA techniques were used [26]. PCR-fragments coding for prepro-PAF (*paf1*), pro-PAF (*paf2*) and mature PAF (*paf3*) were amplified from genomic *P. chrysogenum* DNA by using synthetic oligonucleotides to create an *Nco*I restriction site in the start codons (for prepro-PAF: 5′-GGTACCATGGCCCAAATC-ACCACAGTTG; for pro-PAF: 5′-GGTACCATGGCCACCCCCATTGAGTCTG; for mature PAF: 5′-GGTACCATGGCCAAATACACCGGAGTG) and a *Bam*HI site after the *paf* stop codons (5′-GATCGGATCCCTAGTCACAATCGACAGC). A vector containing the truncated portions of the *paf* gene preceded by the *xylP* promoter and followed by the *A. nidulans trpC* terminator sequence [11] was generated from the plasmids pBluearg and pXylNOM. Cloning of both vectors was described previously by Zadra et al. (2000) [34]. In brief, a 1.7-kb region of the *xylP* promoter was amplified by PCR and subcloned in front of the *uidA* gene into plasmid pNOM102 [25], replacing the *gpdA* promoter and resulting in the plasmid pXylNOM. The *uidA* gene was excised by restriction with *Nco*I/*Bam*HI and replaced by the PCR-fragments *paf1*, *paf2* and *paf3*. Plasmid pBluearg that contained a mutated *argB* allele for single-copy insertion at the *argB* locus of *A. nidulans* WG355 was generated by excision of a 3.7-kb *Nru*I–*Kpn*I fragment from plasmid pTran3-1A [24] and ligation into *Eco*RV/KpnI-cleaved pBluescript KS (Stratagene, La Jolla, CA, USA). The resulting vector pBluearg was digested with *Pst*I and *Bam*HI, ligated with the *Bgl*II/*Pst*I excised expression cassettes carrying the *xylP–paf1*, *xylP–paf2* or *xylP–paf3* fusions as described above and the new transforming vectors were termed pXylpaf1, pXylpaf2 and pXylpaf3, respectively (Fig. 1). The correct reading frame of the PCR fragments was verified by sequencing [26].

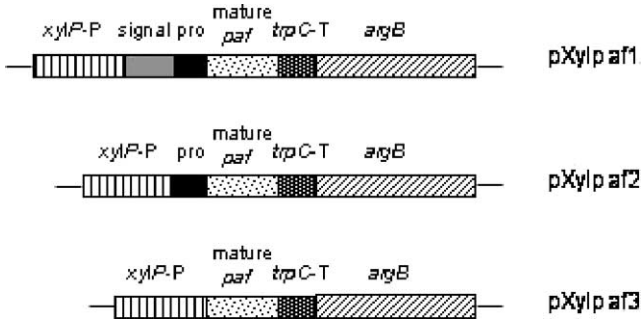


Fig. 1. Schematic representation of the expression vectors pXylpaf1, pXylpaf2 and pXylpaf3 for the complementation of *A. nidulans* strain WG355 (*argB*$^-$) by transformation and for inducible expression of recombinant *paf*. *xylP*-P, *P. chrysogenum*-derived *xylP* promoter; signal/pro/mature *paf*, nucleotide sequence coding for the signal sequence, the prosequence and the mature sequence of *paf*; *trpC*-T, *A. nidulans trpC* termination region; *argB*, *A. nidulans argB* encoding region.

2.3. Transformation of A. nidulans WG355

Transformation of *A. nidulans* WG355 was carried out as described by Tilburn et al. (1995) [29] and resulted in the strains Xylpaf1, Xylpaf2 and Xylpaf3. In order to generate a control strain, *A. nidulans* WG355 was complemented with pBluearg leading to an *argB* reconstituted strain that is further referred to as the wt strain. Fungal DNA and total RNA were isolated from nitrogen-

frozen and ground mycelia as described by Zadra et al. (2000) [34], and screening of positive clones was performed by standard PCR and Southern blot analysis [26]. Digoxigenin-labeled PCR fragments were amplified using oligonucleotides 5′-CCGTTGCGAATGCTAACC and 5′-TCGCATACTCTCCACAATCC for the *argB* probe, 5′-GCCAAATACACCGGAAAATG and 5′-CTAGTCACAATCCCCGACAGC for the *paf* probe, and 5′-CGGTGATGAGGCACAGT and 5′-CGGACGTCGACATCACA for *acnA*. The probes were used for DNA and RNA detection as described by the manufacturer's instructions (Roche, Mannheim, Germany).

2.4. Protein analysis

For recovery of secreted recombinant PAF the supernatants from cultures grown under inducing and repressing conditions for various time points were separated from mycelia by filtration and cleared by centrifugation at 12 000 *g* and 4 °C, prior to 10-fold concentration using Centriprep devices (YM-3, Millipore, Bedford, MA). Total protein content was determined by the method of Bradford (1976) [5]. Equal protein amounts were analyzed by SDS-PAGE (16% polyacrylamide; Novex, San Diego, CA) and immunoblotting, using rabbit anti-PAF serum (1:1000; [20]) as primary antibody and alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (1:10 000; Sigma, Vienna, Austria). The concentrated supernatants were further used in protein activity assays.

To analyze reactive oxygen species (ROS)-induced protein carbonylation, 50 mg of lyophilized mycelia were extracted in 600 µl of 50 mM Na-phosphate buffer, pH 7.0 in a Retsch mixer mill MM 300 (Qiagen, Vienna, Austria). After removal of insoluble cell debris by centrifugation (12 000 *g*, 4 °C), protein carbonyl groups in the soluble cell fractions were derivatized with 2,4-dinitrophenylhydrazine DNPH (Sigma, Vienna, Austria) according to the protocol of Levine et al. (1994) [14]. Samples were separated by SDS-PAGE and proteins were immunodetected by an anti-dinitrophenylhydrazine antibody (1:4000, Dako, Glostrup, Denmark) according to Cabiscol et al. (2000) [7].

2.5. Microscopy

Analysis of hyphal growth and morphology was examined and photographed with an Olympus CK40 microscope (Vienna, Austria), equipped with a Zeiss AxioCam MR (Jena, Germany). For indirect immunofluorescence staining, conidia of transformants were germinated in inducing medium at 30 °C for 16 h. As a control, the wt strain was grown under the same conditions and then treated with 5 µg purified native PAF/ml for 90 min. Indirect immunofluorescence staining for the localization of native and recombinant PAF in *A. nidulans* was performed as described by Oberparleiter et al. (2003) [20]. For the staining of vacuoles, 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein)acetoxymethyl-(AM)ester (BCECF; Molecular Probes, Eugene, OR, USA) was used according to the manufacturer's instructions. Fluorescent-labeled specimens were visualized on a Zeiss 510 confocal laser scanning microscope or alternatively on a Zeiss Axioplan fluorescence microscope (Jena, Germany) as previously described [13].

For transmission electron microscopy (TEM), *A. nidulans* hyphae from liquid cultures were fixed with 2.5% glutaraldehyde in cacodylate buffer CCB (v/v; 21.4 g/l sodium-cacodylate × $3H_2O$, pH 7.3) for 1 h at 4 °C. After washing with CCB for 10 min at room temperature specimens were postfixed in reduced osmium tetroxide (0.5% OsO_4 and 0.75% potassium ferrocyanide in CCB) for 1 h at room temperature. After three washing steps (10 min each) mycelia were dehydrated in standard ethanol series and embedded in Spurr's low-viscosity resin (Plano, Marburg, Germany) for 18 h at 4 °C.

Colonies cultured on solid media were phase-fixed with 2.5% glutaraldehyde and 1% osmium tetroxide in *n*-heptane for 1 h at room temperature. Specimens were then postfixed in osmium tetroxide, washed, dehydrated and embedded as described above. Semithin (300 nm) and ultrathin sections (50–90 nm) were cut with a Leica Ultracut UCT (Vienna, Austria), mounted on regular hexagonal copper grids, double-stained with uranyl acetate and lead citrate and examined with a ZEISS 902 TEM (Jena, Germany).

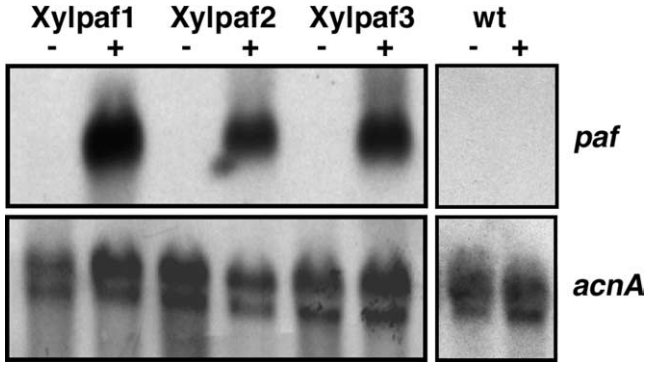
2.6. Statistical analysis

All statistics were done with the Microsoft Excel program and statistical significance was calculated by Student's *t*-test. A two-sided P value $\leqslant 0.05$ was considered to be significant.

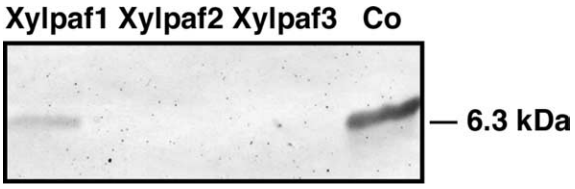
3. Results

3.1. Expression of different recombinant PAF protein types in A. nidulans

A. nidulans was transformed with the reporter constructs as described in Section 2 (Fig. 1). Single-copy transformants with plasmid integration at the *argB* locus were identified by Southern blot analysis in order to determine the impact of the N-terminally truncated PAF protein types (data not shown). Five isolated strains of each transformant type (Xylpaf1, Xylpaf2 and Xylpaf3) with single copy integration were analyzed for any evident phenotype on inducing solid medium. All Xylpaf1 strains showed the same retarded growth and development, whereas the Xylpaf2 and Xylpaf3 isolates did not differ in their appearance from the wt. Subsequently, one isolate of each strain was selected for further characterization. The transcription of the *xylP*-driven *paf* fragments was strongly induced by xylose and completely repressed by glucose in all three Xylpaf strains, which underlines the



(A)



(B)

Fig. 2. Expression of recombinant *paf* and secretion of heterologous protein in the *A. nidulans* strains Xylpaf1, Xylpaf2, Xylpaf3 and wt. (A) Northern analysis was performed with 10 µg total RNA isolated from cultures grown for 24 h under inducing (+) or repressing (−) conditions (upper panel). Detection of *acnA* mRNA was used as loading control (lower panel). (B) Immunoblot analysis of extracellular proteins of transformants grown for 24 h under inducing conditions. 25 µl of 10-fold-concentrated supernatants were loaded per lane. Lanes: 1, Xylpaf1; 2, Xylpaf2; 3, Xylpaf3; 4, wt; co, 0.5 µg purified native PAF was loaded as a control.

efficiency of the expression system (Fig. 2A). The length of *paf* mRNA of the transformants (600, 570 and 510 nt, respectively) corresponded to the length of 620 nt of the native *paf* mRNA (data not shown). No *paf*-specific mRNA was detected in the wt strain that was cultivated under both growth conditions (Fig. 2A). Densitometric standardization of the expression levels revealed similar *paf* mRNA amounts synthesized by the three transformants: $148 \pm 20.9\%$ in Xylpaf1, $136 \pm 17.9\%$ in Xylpaf2 and $124 \pm 27.4\%$ in Xylpaf3, whereby the corresponding *acnA* expression levels were defined as 100%. Further analysis of the transformants was performed under inducing conditions. During induction experiments, the pH of the extracellular medium did not change significantly (data not shown). The secretion of recombinant PAF into the culture supernatant was investigated by SDS-PAGE and immunoblotting. As expected, *A. nidulans* strain Xylpaf1 secreted a protein that reacted with the PAF-specific rabbit antiserum. The molecular weight (MW) corresponded to the MW of 6.3 kDa of native PAF, which had been purified from the *P. chrysogenum* supernatant (Fig. 2B). In the supernatants of *A. nidulans* strains Xylpaf2, Xylpaf3 and wt no PAF-specific signal was found.

The detection of the intracellular portion of the recombinant PAF proteins by immunoblotting of cell-free extracts was not successful, possibly due to little PAF content. Therefore, a more sensitive method was selected, namely indirect immunofluorescence staining. Strong PAF-specific fluorescent signals in the hyphal cells of all three transformants were detected (Fig. 3). In the transformant Xylpaf1 fluorescent signals appeared predominantly in the hyphal tips (Figs. 3A and 3B). The intracellular signal resembled that of the wt that was treated with 5 µg of purified native PAF/ml in inducing medium (Fig. 3G). Pro-PAF and mature PAF accumulated intracellularly in Xylpaf2 and Xylpaf3, respectively, whereby pro-PAF was evenly distributed within the cell (Fig. 3C). Interestingly, mature-PAF accumulated at specific sites in the cytoplasm of not all, but most hyphae (Fig. 3D). However, staining of Xylpaf3 with BCECF excluded a colocalization of recombinant PAF with vacuoles (Figs. 3E and 3F). PAF-specific fluorescent signals were detectable neither in *A. nidulans* transformants which were cultivated under repressing conditions (data not shown), nor in the wt strain which was grown under inducing or repressing conditions (Fig. 3H). These controls proved the PAF-specificity of the immunofluorescence staining.

3.2. Effects of recombinant PAF protein types on fungal growth

In order to study the effect of recombinant PAF production on the growth of Xylpaf1, Xylpaf2 and Xylpaf3, inducing or repressing solid and liquid media were inoculated with the corresponding spores. Xylpaf1 grew significantly more slowly on inducing solid MM than the strains Xylpaf2, Xylpaf3 and wt (Fig. 4A). Additionally, conidiation of the Xylpaf1 strain was retarded during this period of time. However, after 7 days of further incubation differences in colony size were still evident, but Xylpaf1 had started to conidiate. No significant differences either in the number or in the germination efficiency of the produced conidia were detectable between Xylpaf1 and the other *A. nidulans* strains tested (data not shown). Comparable results were obtained when colony formation and development of the transformants were monitored on solid CM, indicating that the phenotypes were expressed independently of the medium composition (data not shown). All *A. nidulans* strains grown on repressing glucose containing solid media grew better than on inducing xylose-containing media, and colony diameters and conidiation were similar at any time point under repressing conditions (Fig. 4B).

Comparable results were obtained when mycelial growth in inducing liquid medium was determined by light microscopy and spectrophotometry. However, these methods revealed that not only the growth of Xylpaf1, but also that of Xylpaf3, was affected under inducing conditions. Conidia of all transformants germinated at similar rates, but hyphal growth of strains Xylpaf1 and Xylpaf3 was retarded with further incubation, as determined by light microscopy (Fig. 5). Xylpaf1 showed the most prominent phenotype with short multiply curved hyphae and numerous small ramifications, indicating not only growth retardation but also a defect in hyphal morphology (Figs. 5A and 5F). The hy-

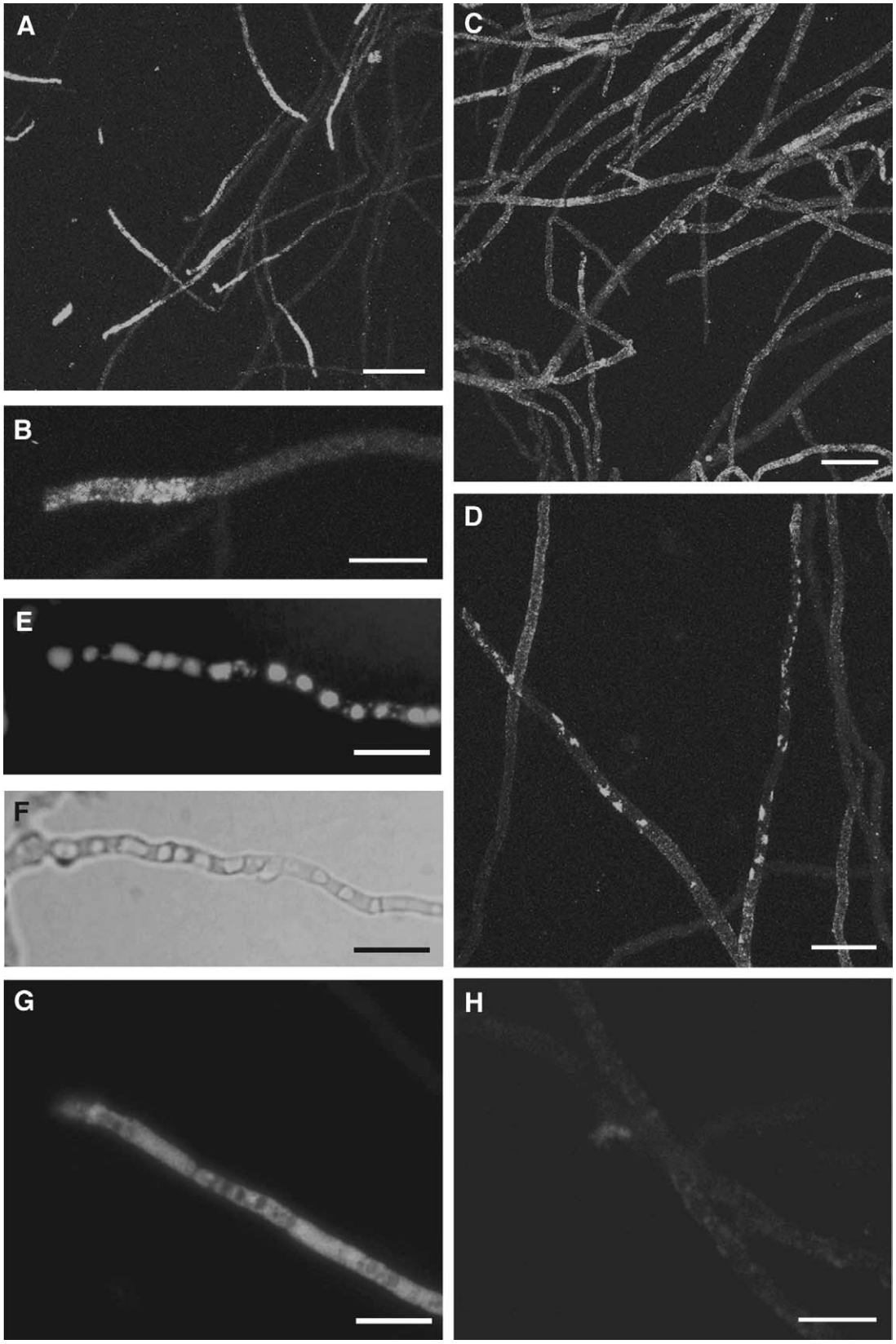
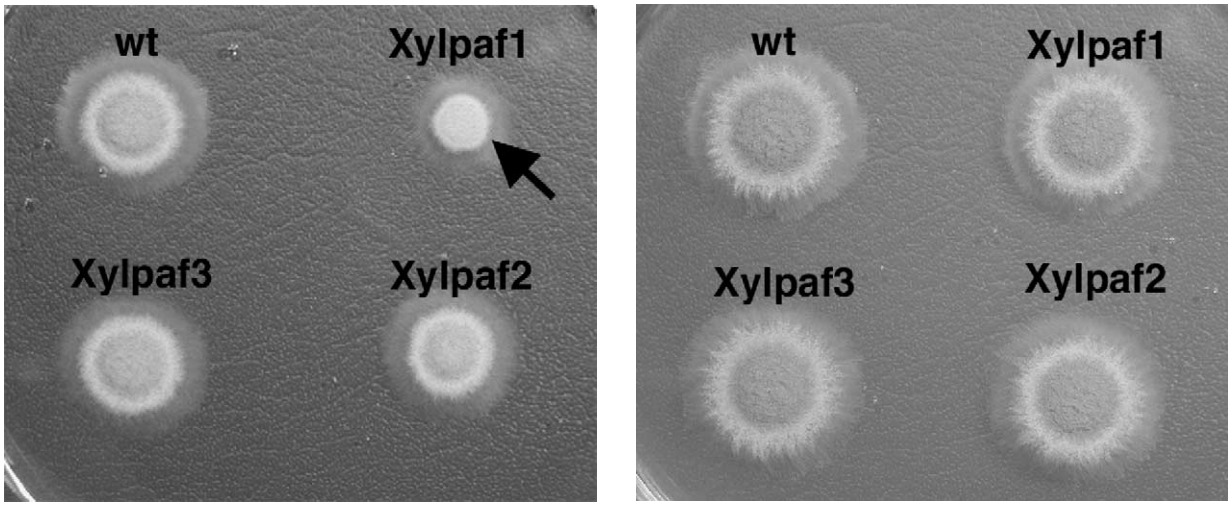


Fig. 3. Fluorescence microscopy of *A. nidulans* transformants, expressing recombinant PAF. The localization of recombinant PAF proteins was performed by indirect immunofluorescence staining of *A. nidulans* transformants, which were grown in inducing medium for 24 h. (A), (B) Xylpaf1, (C) Xylpaf2, (D) Xylpaf3 and (H) wt. (G) *A. nidulans* wt, treated with 5 μg PAF/ml for 90 min in inducing medium was used as a positive staining control. (E) Vacuoles of Xylpaf3 labeled with the fluorescence dye BCECF revealed a different staining pattern compared to the PAF specific signals in Xylpaf3, (F) light microscopic control of (G). Scale bars: 30 (A), 10 (B, E, F, G, H), 20 (C), 15 μm (D).



(A) (B)

Fig. 4. Growth of the *A. nidulans* strains Xylpaf1, Xylpaf2, Xylpaf3 and wt after 72 h on inducing solid medium. (A) The colony diameter of the transformant Xylpaf1 (arrow) was significantly smaller on inducing medium compared to that of the other transformants or the wt strain. (B) As a control, transformants were grown under repressing conditions.

phae of Xylpaf1 strongly resembled the hyphae of the wt which were exposed to 1 µg of purified native PAF/ml under the same inducing growth conditions (Figs. 5E and 5J). This underlines the fact that the phenotypic characteristics of Xylpaf1 derive from the expression of a functional, recombinant PAF protein. Instead, the hyphae of Xylpaf3 were longer, less curved and formed more regular branches (Figs. 5C and 5H). Both Xylpaf2 and the wt grew in long straight hyphae with regular branch formation (Figs. 5B, 5G, 5D and 5I). Measurement of the OD_{620} of fungal cultures underlined the growth retardation that had been observed microscopically. 24 h after inoculation, Xylpaf1 showed $19.6 \pm 4.1\%$ growth ($P \leqslant 0.01$), Xylpaf2 $113.3 \pm 7.1\%$ and Xylpaf3 $77.2 \pm 8.2\%$ ($P \leqslant 0.45$) compared to the wt strain, whose growth was defined as 100%. However, spectrophotometric quantification of hyphal growth revealed less sensitivity compared to microscopic analysis. Although marked growth retardation of Xylpaf3 was evident by light microscopy, the 23% growth inhibition of Xylpaf3 which was calculated from the determined OD_{620} was not significantly different compared to the control. After 72 h of incubation, growth resulted in $37.5 \pm 4.3\%$ for strain Xylpaf1 ($P \leqslant 0.02$), $96.2 \pm 6.9\%$ for strain Xylpaf2, and $71.8 \pm 1.7\%$ for strain Xylpaf3 ($P \leqslant 0.15$) compared to the 100% growth of the wt strain. In glucose containing liquid medium all strains proliferated similarly at any time point (data not shown).

The functionality of the recombinant PAF secreted by the transformant Xylpaf1 was tested also on the highly sensitive mold *A. niger* in a microtiter plate assay. The supernatant of Xylpaf1 severely inhibited the growth of *A. niger*, whereas no growth retardation was detectable with the supernatants of Xylpaf2, Xylpaf3 and that of the wt strain (Fig. 6). The activity of the Xylpaf1 supernatant was compared to the activity of native PAF which had been serially diluted in conditioned supernatants of the strains Xylpaf2, Xylpaf3 and wt. The protein activity in the supernatant of Xylpaf1 corresponded to 0.1–0.5 µg of purified native PAF/ml, since the growth inhibition induced by the 10-fold concentrated culture supernant was equivalent to that induced by 1–5 µg PAF/ml (Fig. 6).

The induction of ROS was analyzed by the detection of oxidatively damaged proteins in the transformants grown under PAF-inducing conditions. As shown in Figs. 7A and 7B a marked increase in protein carbonylation of soluble extract of Xylpaf1 was evident, compared to that of Xylpaf2, Xylpaf3 and of the wt strain indicating that Xylpaf1 indeed suffers from oxidative stress due to the activity of recombinant PAF.

3.3. Cellular ultrastructure of PAF-expressing A. nidulans

To investigate possible effects of PAF on the cellular ultrastructure of the transformants, ultrathin hyphal sections were monitored by transmission electron microscopy (Fig. 8). *A. nidulans* wt hyphae featured cells with regular submicroscopic structure and clearly identifiable organelles (Fig. 8A). Hyphal segments typically contained several nuclei surrounded by the nuclear membrane. Nucleoli were visible in many cases. The numerous mitochondria with membranous cristae always showed well preserved outer and inner membranes. Vacuoles were empty or contained loosely distributed material. The fungal cytoplasmic membrane was visible as a sharp, electron-dense lipid bilayer, surrounded by the cell wall with an inner finely granular and an outer fibrous layer. Xylpaf2 exhibited a similar subcellular morphology (Fig. 8E).

In contrast, significant differences were evident in the Xylpaf1 strain (Fig. 8B and 8C). Hyphae showed an increased number of small vacuoles which contained com-

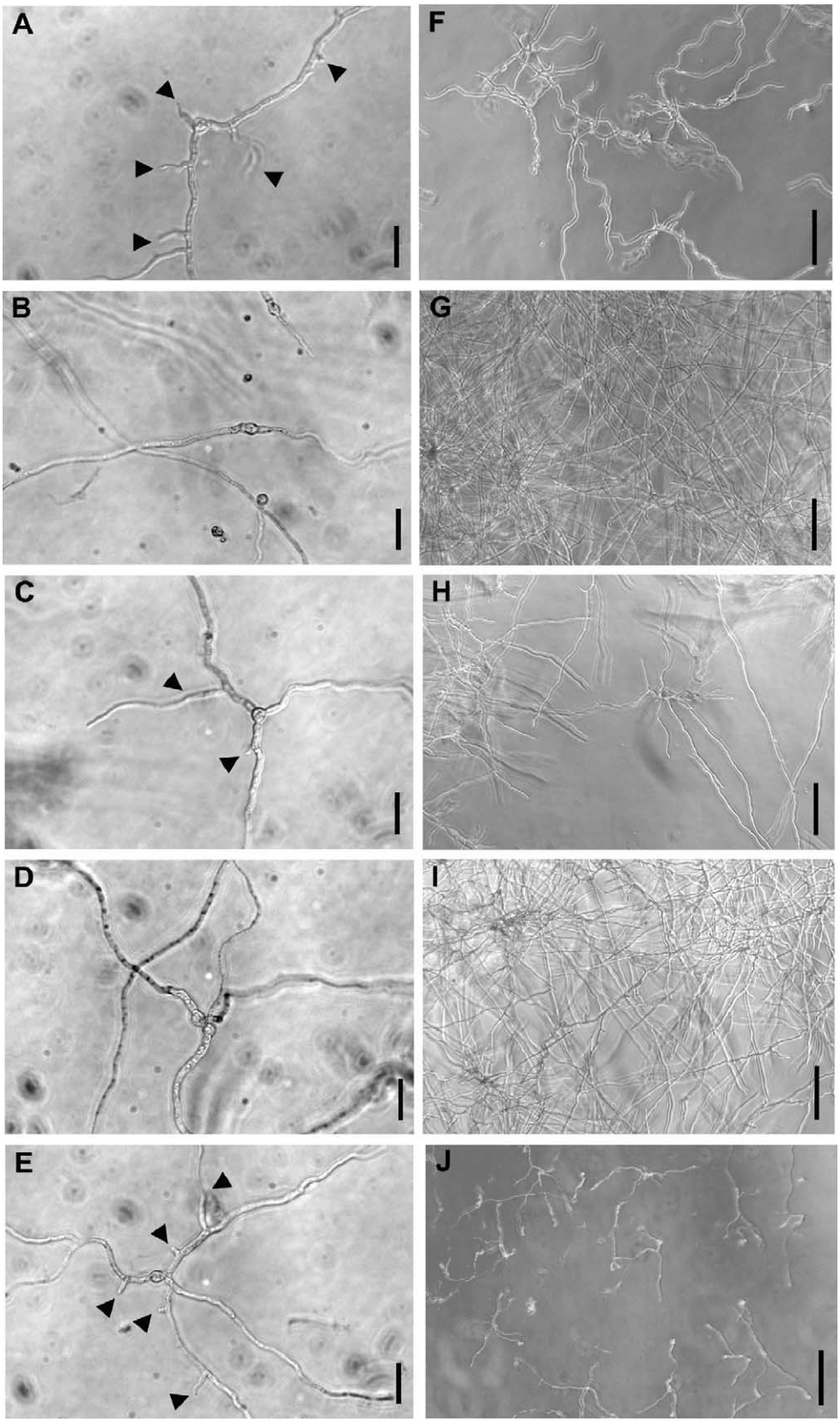


Fig. 5. Light micrographs of *A. nidulans* strains Xylpaf1, Xylpaf2, Xylplaf3 and wt cultivated in inducing liquid medium for 24 h (A–E) and 40 h (F–J). The detail and the general view of the cultures indicate growth retardation and changes in growth polarity of (A, F) Xylpaf1, (C, H) Xylpaf3 and (E, J) wt, treated with 1 μg purified native PAF/ml compared to normal growth of (B, G) Xylpaf2 and (D, I) wt. Multiple branch formation is indicated by arrowheads. Scale bars: 20 (A–E); 100 μm (F–J).

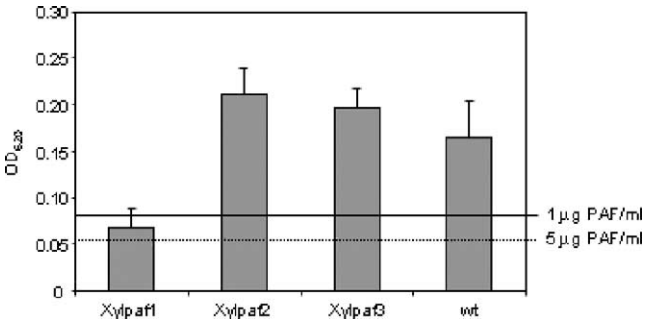


Fig. 6. Spectrophotometric determination of the growth of *A. niger* by measuring the OD_{620} in microtiter plates after 48 h of incubation in CM. The growth inhibitory activity of the 10-fold concentrated supernatants of the *A. nidulans* strains Xylpaf1, Xylpaf2, Xylpaf3 and wt were compared with the activity of purified native PAF in conditioned supernatant (—) 1; (···) 5 µg PAF/ml.

pact, electron-dense inclusions. In general, mitochondria were less well defined. In hyphae from liquid cultures, the outer mitochondrial membrane was most often discontinuous or missing (Fig. 8B). Mitochondrial profiles appeared connected to each other in the majority of the cells. In hyphae from solid cultures, outer mitochondrial membranes were distinct. Compared to the wt, a much higher number of mitochondria showed conspicuous cytoplasmic indentations (Fig. 8C). These morphological alterations were PAF-specific, since the morphology of Xylpaf1 specimens derived from repressing growth conditions was comparable to that of the wt (Fig. 8D).

The cellular ultrastructure of strain Xylpaf3 exhibited no massive aberrations and resembled strain Xylpaf2 and the wt strain, with the exception of the mitochondria. These

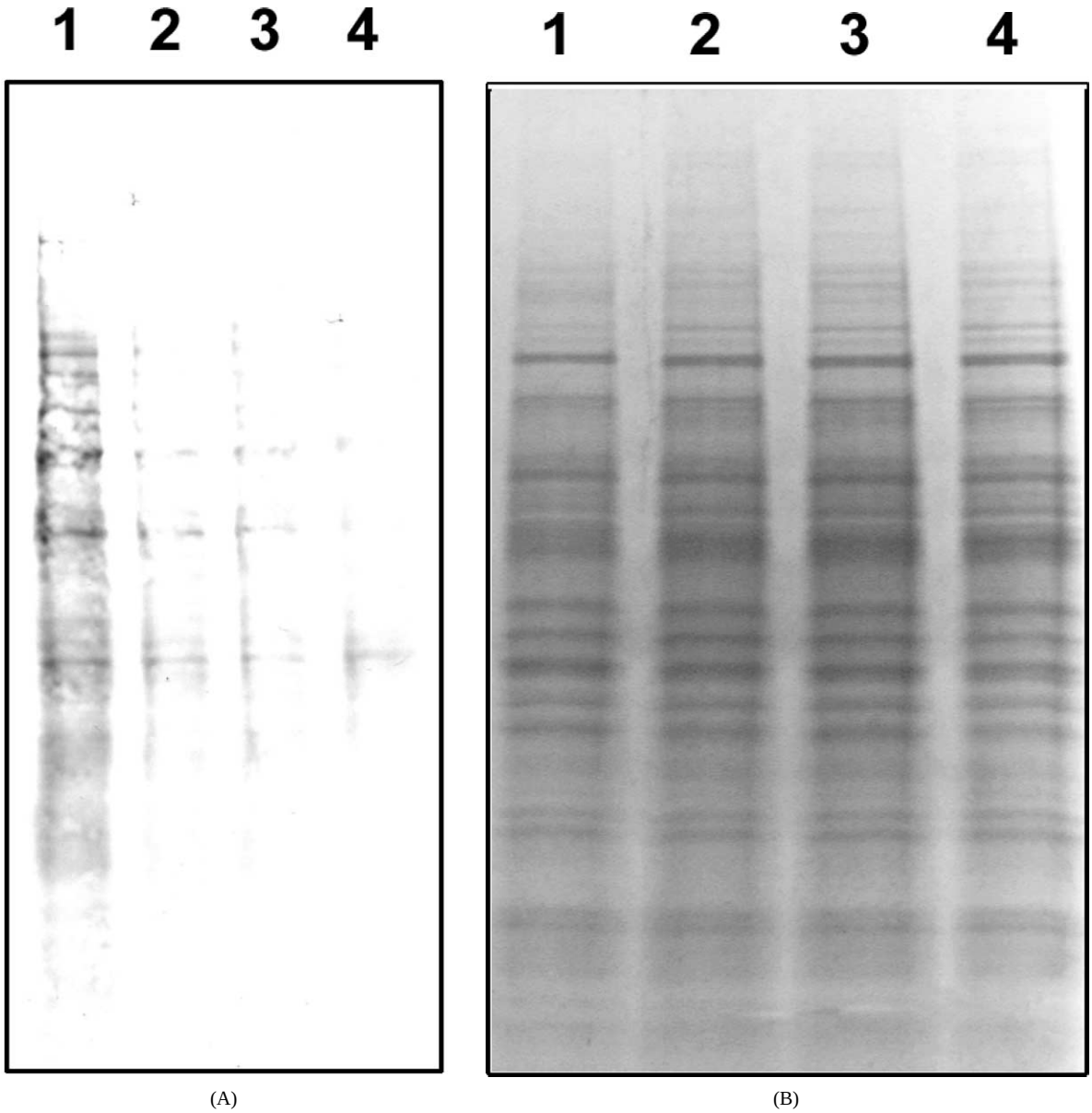


Fig. 7. Pattern of carbonylated proteins of cellular extracts of *A. nidulans* strains cultivated in inducing liquid medium for 24 h. (A) Anti-2,4-dinitrophenol immunostain; (B) Coomassie-brilliant blue protein stain as loading control. Lanes: 1, Xylpaf1; 2, Xylpaf2; 3, Xylpaf3; 4, wt.

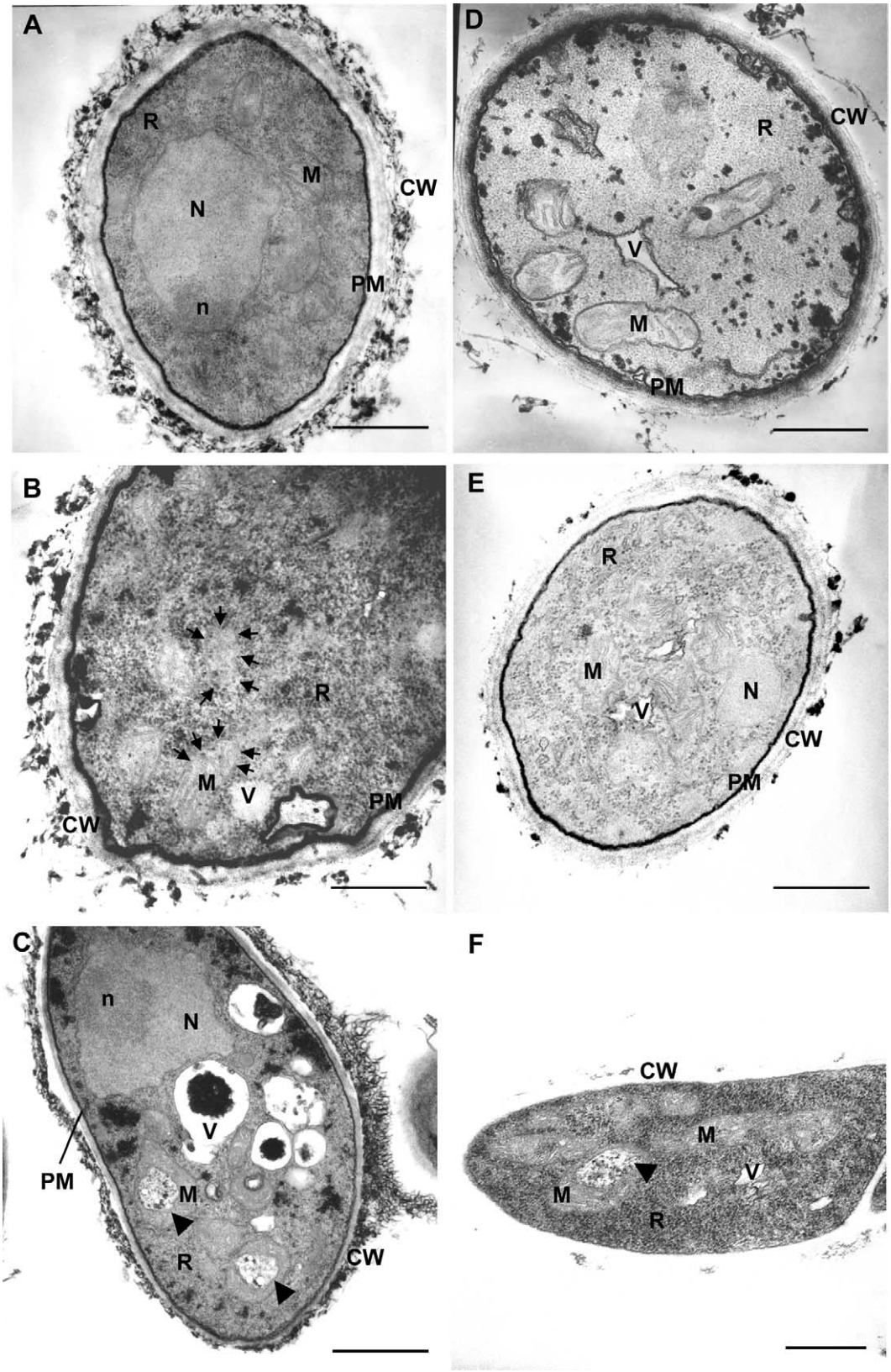


Fig. 8. Transmission electron micrographs of the cellular ultrastructure of *A. nidulans* strains cultivated in inducing liquid (A, B, E, F) and solid medium (C), and in repressing liquid medium (D) for 24 h, respectively. (A) wt, (B, C, D) Xylpaf1, (E) Xylpaf2, (F) Xylpaf3. Mitochondria with discontinuous outer membrane are marked with small arrows, mitochondria with cytoplasmic indentations are marked with arrowheads. CW, cell wall; P, peroxisome; M, mitochondrium; N, nucleus; n, nucleolus; PM, plasma membrane; R, ribosomes; V, vacuole. Scale bars: 0.5 (A, B, D, E); 1.3 (C); 0.6 μm (F).

organelles were similarly affected as in Xylpaf1, both in hyphae of liquid (Fig. 8F) and solid cultures (data not shown). However, these alterations were seen more rarely.

4. Discussion

Analysis of EST and genomic sequence data bases revealed the absence of a *paf* homologous gene in *A. nidulans* [16]. Additionally, *A. nidulans* WG355 which was used in the present study revealed the same PAF sensitivity (data not shown) as *A. nidulans* FGSC A4, a test strain previously used for the characterization of purified, native PAF [13,20]. These facts indicate that this PAF-sensitive mold is an ideal model organism for investigating the effects of heterologous expression of N-terminally truncated PAF proteins upon its growth, physiology and morphology. The recombinant *paf* genes were efficiently transcribed at similar levels in the respective transformants under inducing conditions, whereas no *paf* mRNA could be detected in the wt. In Xylpaf1 the recombinant protein was secreted into the supernatant and was found to be functional, whereas no PAF-specific signals were detectable in the supernatants of *A. nidulans* strains Xylpaf2, Xylpaf3 and the wt. This result is in accordance to the expectation that neither pro-PAF nor mature PAF is secreted due to the lack of a signal sequence. However, the amount of the corresponding recombinant PAF proteins in the supernatant and in the cellular extracts was very low. Self-inhibition of proliferation of Xylpaf1 under PAF-inducing growth conditions and increased degradation of recombinant pro-PAF and mature PAF in Xylpaf2 and Xylpaf3 might have contributed to the poor protein yield. Alternatively, insufficient protein translation in all strains could be attributed to incorrect pre-mRNA processing, a suboptimal translation start region or limitations at the post-translational level [10].

Characterization of the tranformants Xylpaf1, Xylpaf2, Xylpaf3 and of the wt strain under PAF-inducing conditions revealed significant differences in the growth rates in liquid as well as solid media, in the intracellular localization of PAF and in the hyphal morphology and cellular ultrastructure. Xylpaf1 was the most affected strain, showing phenotypic and physiological characteristics which were equally induced in wt hyphae by the addition of purified native PAF, and which had been recently reported for PAF-treated *A. nidulans* FGSC A4, e.g., growth retardation and multiple branch formation [13]. We also showed in a previous study that the toxicity of PAF depends on its intact structure and on its transport into *A. nidulans* hyphae [20]. The predominant accumulation of the immunofluorescence signal in the hyphal tips of Xylpaf1 does not enable discrimination between protein secretion and uptake, since both processes take place in this part of the hyphae [9,18,30,33]. However, since PAF was found in the supernatant it can be assumed that it was efficiently secreted. On the other hand, the affected phenotype of Xylpaf1 points to the uptake of a properly processed PAF. However, expression of recombinant PAF in Xylpaf1 was not lethal and growth and morphology of Xylpaf1 were less affected than *A. nidulans* wt cells, which were exposed to purified native PAF [13]. This can be explained by the relatively small amount of produced recombinant protein whose concentration was well below the level (> 50 μg/ml) which is necessary to kill hyphal cells [13].

One still unsolved question is the cause for growth inhibition. Due to the high specificity of PAF and the fact that this antifungal protein is internalized by sensitive fungi, it can be hypothesized that target molecules are not only present on the outer fungal layers, which might confer selectivity for this protein, but also reside intracellularly, where detrimental effects might be evoked. In fact, in a recent study we revealed PAF-induced generation of intracellular ROS in *A. niger* [13], as well as in *A. nidulans* (paper in preparation). Oxidative damage of macromolecules occurs in cells at a background level despite adaptive responses. Under increased oxidative stress conditions, cells exhibit a lower viability, because ROS damage proteins, lipids and nucleic acids, and are deleterious to mitochondria. Finally, this process affects the metabolic and structural integrity of the cell [7]. Xylpaf1 showed several signs of elevated oxidative stress during expression of recombinant PAF: an increased level of protein carbonylation, changes in mitochondrial morphology and severe growth retardation. Although we are thus far unable to discriminate between a primary and a secondary role of PAF in causing oxidative stress, this mechanism seems to play a major role in the effectiveness of this antifungal protein.

PAF is translated as prepro-protein and the signal sequence for secretion as well as the prosequence is removed before or during the release of mature PAF into the supernatant [17]. There are various examples of similarly processed antimicrobial proteins, e.g., *A. giganteus* antifungal protein AFP [32] and ribotoxin α-sarcin [21,31], killer toxins from *Saccharomyces cerevisiae* [27] and from *Ustilago maydis* [28], and *Streptomyces tendae* chitin binding protein [4]. Generally, prosequences were attributed the role of "intramolecular chaperones", which prevent proteins from exerting an activity before secretion, or which maintain proteins unfolded to ensure their interaction with the transport machinery of the cell [8]. Intracellular expression of pro-PAF in strain Xylpaf2 resulted in a phenotype identical to the wt, although its cytoplasmic localization resembled that of internalized native PAF [20]. Therefore, it can be assumed that the prosequence prevented PAF from premature activity. A similar function was reported for the prosequence of the *Rhizopus oryzae* lipase, which (1) modulates enzyme activity in such a way that the enzyme can initially be synthesized in a non-destructive form, and (2) supports folding and disulfide bonding [1,2].

In contrast, Xylpaf3 expressed PAF intracellularly without signal and prosequence. The expression of mature PAF slightly impaired the proliferation of Xylpaf3, which points to weak but still detectable protein activity. However,

changes in mitochondrial morphology were minimal and no elevated protein carbonylation was detectable. Remarkably, the distribution of the intracellular immunofluorescent signal differed from that of Xylpaf1, Xylpaf2 and of internalized native PAF [20]. The numerous immunofluorescent patches in Xylpaf3 might originate from aggregated misfolded protein due to the missing support of the prosequence for proper folding. Alternatively, a mislocalization of the protein could prevent its interaction with putative target molecules and lead to increased degradation and inefficient protein activity. Since we previously showed that native PAF was localized cytoplasmically in sensitive molds [20], we expressed in this study recombinant pro-PAF and mature PAF intracellularly to investigate their activity at the putative site of action. However, there was no or only a minor effect detectable in the phenotypes of Xylpaf2 and Xylpaf3 compared to the wt. Therefore, it cannot be excluded that PAF has to pass the cell wall and/or the plasma membrane to become fully active. The expression of a recombinant pre-mature-PAF, lacking the prosequence might provide the answer to this question and will be the subject of future studies.

In this respect, we tried to express in another approach mature PAF in a prokaryotic expression system, using the plasmid vector pET3d in both, the *Escherichia coli* strains BL21 and Origami™. The latter strain greatly enhances disulfide bond formation of recombinant proteins in the cytoplasm [3,23]. Although large amounts of recombinant protein were purified from both *E. coli* strains, recombinant mature PAF was inactive (unpublished data). This again underlines the necessity of prosequence-supported protein folding and proper protein processing for PAF activity.

In conclusion, the results presented in this study indicate that correct protein processing and proper folding is necessary for PAF activity and that the prosequence seems to play an important role in preventing PAF from premature activity. However, projected immunogold labeling and transmission electron microscopy will address the subcellular localization of recombinant PAF in more detail. Additionally, crystallographic analysis might elucidate the structural relationship between the prosequence and the mature protein, since no information on the three-dimensional structure of PAF involving its prosequence is available to date.

Acknowledgements

We thank Hubertus Haas, Bernhard Redl and Gunde Rieger for helpful discussions and the reading of the manuscript. This work was funded by the Austrian Science Foundation (grant FWF P15261 to F.M.).

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