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Short communication

The *Cladosporium fulvum Bap1* gene: evidence for a novel class of Yap-related transcription factors with ankyrin repeats in phytopathogenic fungi

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Abstract

The *Saccharomyces cerevisiae* transcription factor gene *YAP1* is a key regulatory gene conferring resistance against oxidative stress and various cytotoxic compounds. It would be of interest to isolate homologous genes in pathogenic fungi as candidate pathogenicity genes. We have developed a generally applicable hybridization method, using a single degenerate oligonucleotide probe designed on the basis of the conserved DNA-binding region of yAP1 homologs in yeast species, to clone a related gene, *Bap1,* from the tomato pathogen, *Cladosporium fulvum.* This gene has the capacity to code for a possible transcription factor consisting of an N-terminal, yAP1-related, basic DNA-binding domain. It lacks an obvious downstream leucine zipper but includes C-terminal ankyrin-like repeats. The *Cochliobolus carbonum* pathogenicity factor TOXEp shows a similar modular structure. BAP1 and TOXEp appear to represent a novel fungus-specific class of Yap basic DNA-binding domain-type transcription factors with ankyrin-like repeats (bANK proteins) in phytopathogenic fungi. A possible role of BAP1 in a membrane transporter-mediated detoxification or secretion process is discussed.

Abbreviations: ANK – ankyrin-like; bZIP – basic region-leucine zipper.

Successful pathogens have to defend themselves against host defence reactions, adverse environmental conditions and, in some cases, fungicides administered by man to control pathogen growth. In this context, the roles of the oxidative-stress response (Lamb and Dixon, 1997) and membrane-efflux detoxification systems (Del Sorbo et al., 2000) in phytopathogenic and medically important fungi are currently a very active area of research. It would be of great interest to identify regulatory genes of these fungal defence systems as candidate pathogenicity genes.

The *Saccharomyces cerevisiae YAP1* gene encodes a basic region-leucine zipper (bZIP)-type transcription factor, yAP1, which has been identified as a key stress-response regulator, conferring resistance to oxidative stress and a variety of cytotoxic compounds (Moye-Rowley et al., 1989; recently reviewed by Toone and Jones, 1999). Genes under its control encode, for example, glutathione reductase, thioredoxin and several membrane transporters. yAP1 is the founding member of the fungus-specific family of bZIP proteins, Yap proteins, defined by four atypical

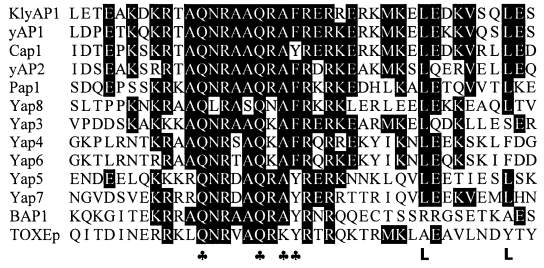

Figure 1. Similarity between the putative basic DNA-binding regions of yeast Yap proteins, BAP1 and TOXEp. The QxxxxQxA(F/Y) motif characteristic of Yap basic DNA-binding domains is indicated (♣). The position of the first two conserved leucines (or other residues) of the leucine zippers in the yeast proteins is also indicated (**L**).

residues on the DNA binding surface (Fernandes et al., 1997).

The sequences of yAP1 and functional homologs in yeast species, *Schizosaccharomyces pombe* Pap1 (Toda et al., 1991), *Candida albicans* Cap1 (Alarco et al., 1997) and *Kluyveromyces lactis* KlyAP1 (Billard et al., 1997) display a high degree of sequence conservation only in the basic DNA-binding region (Figure 1). This fact and also the apparently low expression levels of Yap proteins may complicate various commonly used cloning procedures. The possibility of isolating filamentous fungal *YAP1* homologs was explored using a single degenerate oligonucleotide probe mixture. The 11 amino acids consensus motif AQNRAAQRA(F/Y)R is present in yAP1, the three functional homologs and yAP2 (Wu et al., 1993). All these proteins also have a C-terminal cysteine-rich domain functioning as a putative redox-sensitive nuclear export signal. Importantly, the consensus sequence discriminates against the distantly related Yap proteins, Yap3-8, *Aspergillus nidulans* MEAB (Polley and Caddick, 1996) and non-Yap bZIP transcription factors.

A 64-fold degenerate 32-mer oligonucleotide probe including six deoxyinosine residues (dGCICARAAYMGIGCIGCICARMGIGCITTYMG) based on the 11 amino acids consensus motif (omitting tyrosine codons at position 10) was 5′ end-labelled with T4 polynucleotide kinase. It was used to screen a genomic library (Bussink and Oliver, 2001) of the tomato pathogen, *Cladosporium fulvum* (Oliver et al., 2000), under previously described hybridization stringencies (Bussink et al., 1991). Ten strongly hybridizing phages were found amongst 32,000 plaques. Similar frequencies were observed in other gene-specific hybridizations with long gene fragments as the probe. Nine plasmids were excised from these positive phages and analyzed on Southern blots. Eight plasmids showed the same ∼4 kb hybridizing *Hinc*II fragment, indicating that the inserts contained the same gene. The hybridizing sequence was subsequently located on a 0.8 kb *Sph*I-*Pvu*II fragment that was subcloned. The gene, designated *Bap1*, was sequenced using the subclone and genomic clones (Figure 2A). The nucleotide sequence hybridizing with the probe was identified and one mismatch was found between the probe and gene sequences, at position 29 in the probe. This position corresponds to the phenylalanine or tyrosine residues at position 10 of the consensus motif. The *Bap1* gene shows a tyrosine codon (TAT) in agreement with the consensus motif but only phenylalanine codons (TTY) had been included in the probe. This observation indicates that the hybridization conditions employed were rather permissive, whilst specific for a single *C. fulvum* gene. These conditions should thus also be of use to clone genes coding for a conserved yAP1 DNA-binding domain from other fungi. Additionally, five cDNA clones were isolated from a previously described library (Nielsen et al., 2001).

Comparison of the genomic and cDNA sequences indicated the presence of two introns in the gene. One of the cDNAs appeared to represent a partially processed mRNA species as it lacked a polyA tail and only the first intron had been removed. It is noted that the first intron is located in the extreme 3′ end of the sequence hybridizing with the oligonucleotide probe but that this, coincidentally, would not affect probe hybridization.

The *Bap1* gene has the capacity to code for a putative protein of 157 amino acids, BAP1, showing the 11 amino acids motif characteristic of functional *YAP1* homologs, including the four Yap-specific amino acids (Figures 1 and 2A). It can be seen that the latter proteins have additional conserved residues not found in BAP1. The BAP1 sequence conforms to the bZIP transcription factors' basic domain PROSITE motif. By contrast, BAP1 shows no obvious downstream leucine zipper. It also lacks a highly related Yap-like C-terminal cysteine-rich domain, which might suggest that *Bap1* is not a functional *YAP1* homolog. However, the putative 157 amino acids gene product has five cysteines, including a Cys-xx-Cys motif found in metal binding and redox proteins (McRee, 1998). The C-terminal part of the putative translation product shows a region of

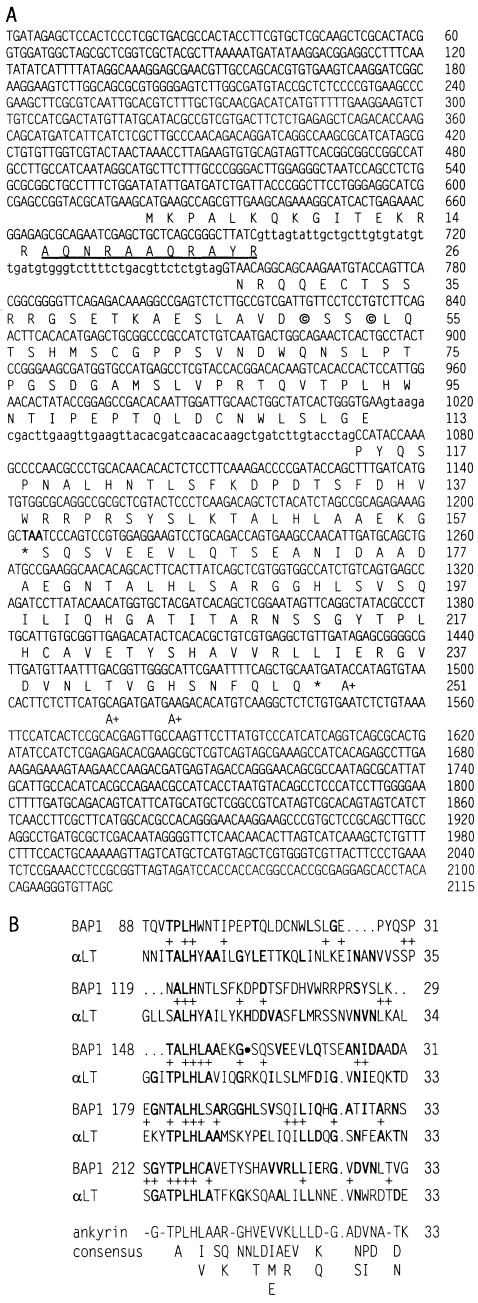

Figure 2. Structure of the *Bap1* gene. (A) Nucleotide and deduced amino acid sequences of the *Bap1* gene. The amino acid sequence characteristic of functional *YAP1* homologs in yeast species is *underlined* and cysteines in a possible metal binding motif are indicated (©). Intron sequence is in *lowercase* and the start of polyA tails in 4 cDNAs (the third site was found twice) is indicated below the sequence (A+). The first translation termination codon is in *bold*. The longest sequenced cDNA started at position G146. (B) The C-terminal part of BAP1 shows ankyrin repeats. The C-terminal sequence of BAP1 was aligned with α-latrotoxin sequence (Kiyatkin et al., 1990) and identical residues are indicated (+). Residues that conform to the consensus sequence of ankyrin repeats are in bold and the length of the repeats is given on the right. The consequence of ochre codon 158 is represented by a symbol (•). GenBank accession number AF288532.

ankyrin-like (ANK) repeats (Figure 2B). ANK repeats mediate protein–protein interactions and are usually 33 residues long (Michaeli and Bennett, 1992). Some ANK repeat proteins consist of only two copies of the ANK repeat but the majority of proteins have at least four copies (Zhang and Peng, 2000). The third repeat in BAP1 is interrupted by an ochre codon (this codon was repeatedly confirmed in genomic and cDNA sequences). The presence of two additional repeats following the ochre codon may appear cryptic, in particular, because these repeats show higher similarity to the ANK consensus sequence than the upstream repeats. One intriguing possibility is that inefficient termination of translation at the ochre codon (Steneberg et al., 1998; Bertram et al., 2001) may result in the synthesis of two BAP1 proteins of 157 or 251 amino acids with two or five ANK repeats, respectively.

The putative product of the *C. fulvum* gene isolated here, BAP1, is characterized by a modular structure of an N-terminal highly yAP1-related basic DNA-binding domain lacking an obvious downstream leucine zipper, and ANK repeats at the C-terminus (bANK protein). No homologous gene is present in the *S. cerevisiae* genome and no other homologs were found in database searches. An additional search with the nonapeptide AQNRAAQRA revealed a possible *Neurospora crassa* functional *YAP1* homolog, which was recently identified in the German genome sequencing project (GenBank CAB91681).

ANK repeats have previously been observed in several transcription factors, but not in Yap proteins. The *Cochliobolus carbonum* pathogenicity factor, TOXEp, was described as a protein with an N-terminal bZIP basic DNA-binding motif with no discernible leucine zipper, but with four ankyrin repeats at its C-terminus (Ahn and Walton, 1998). Its DNA-binding region is clearly Yap related, showing three out of the four residues characteristic of Yap proteins (Figure 1). The structures of BAP1 and TOXEp may thus indicate that the Yap DNA-binding domain has been incorporated into at least two novel classes of transcription factors during fungal evolution. One class consists of the

classical Yap-type bZIP factors; the other class consists of the bANK proteins BAP1 and TOXEp, both occurring in phytopathogenic fungi. Alternatively, bANK proteins might be considered as a novel distinct subclass of Yap proteins. In any case, such an evolutionary origin within the fungal lineage would explain the apparent rareness of bANK proteins, which might make these factors especially attractive to study fungus-specific processes in relation to pathogenicity. In particular, the function(s) of the ANK repeats in these proteins remain to be investigated (Ahn and Walton, 1998).

TOXEp is a pathway-specific transcription factor required for HC-toxin production and pathogenicity and is only present in HC-toxin producing isolates. The biotrophic pathogen *C. fulvum* is not known to produce phytotoxins. The isolation of *Bap1* thus raises the possibilities that bANK proteins may be conserved in phytopathogenic fungi and function in processes other than toxin synthesis. In fact, TOXEp is not required for activity of the central enzyme in HC-toxin biosynthesis (cyclic peptide synthetase). Instead TOXEp is required for the expression of three genes including *TOXA* coding for a putative HC-toxin efflux pump, which is thought to contribute to self-protection against the toxin (Pitkin et al., 1996). The role of yAP1 factors in transporter-mediated resistance to cytotoxic compounds in yeasts is well established. The similarity in the DNA-binding regions of yAP1 and BAP1, in particular, might suggest that these factors could regulate some similar genes, for example, genes involved in the oxidative-stress response or membrane transporter genes. A hypothetical BAP1-regulated transporter might function to remove damaged molecules originating from the oxidative burst upon pathogen recognition, or to protect the fungus from toxic substances produced by the plant host (e.g. tomatine), or to secrete an unidentified fungal secondary metabolite or peptide. Disruption of the *Bap1* gene would help resolve these possibilities.

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