Signaling of Ambient pH in Aspergillus Involves a Cysteine Protease*

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In Aspergillus nidulans, the regulation of gene expression in response to changes in ambient pH is mediated by the PacC zinc finger transcriptional regulator. At alkaline ambient pH, PacC is proteolytically processed to a functional form serving as an activator of alkaline-expressed genes and a repressor of acid-expressed genes. This activation of PacC occurs in response to a signal mediated by the products of the pal genes. Thus, the products of the palA, -B, -C, -F, -H and -I genes constitute an alkaline ambient pH signal transduction pathway. How the pal signal transduction pathway senses ambient pH and transduces a signal to trigger PacC processing is a fascinating unresolved problem. We have cloned and sequenced the palB gene and shown that the predicted PalB protein has sequence similarity to the catalytic domain of the calpain family of calcium-activated cysteine proteases. Thus, a cysteine protease is a component of the alkaline pH signal transduction pathway in Aspergillus.

MATERIALS AND METHODS

A. nidulans Strains and Media—A. nidulans strains carrying markers in standard use (6). Standard media were used (1, 3, 7). palB Cloning and Gene Libraries—A chromosome-located A. nidulans pWE15 cosmid library (8) was used. A strain of genotype pabaA1 argB2 araA49 chaA1 palB7 was cotransformed with pools of chromosome VII cosmid clones and the argB-containing plasmid plJ16 (9). Transformations were carried out as described previously (3). argB transformants were tested for growth on pH 8 medium (1, 5).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank* (7)*EMBL Data Bank with accession number(s) Z54244.

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**FIG. 1. Partial restriction map of palB genomic region, fragments that transform palB7 strains to pal- and palB gene disruption.** A, restriction map and transforming fragments. S, Sphl; X, XbaI; R, EcoRI; B, BamHI; K, KpnI. The striped bar indicates the XbaI/KpnI fragment used to probe Northern blots and screen for palB cDNAs; the EcoRI/BamHI fragment contained within it was also used as a probe for Northern blots and in cDNA screening. The BamHI and Sphl fragments above the map both transform palB7 strains to pal-, but the Sphl fragment does so at a higher frequency. B, palB gene disruption. The arrow indicates the direction and location of the palB transcriptional unit. For disruption with a linear restriction fragment, a pyr4-containing BglII fragment was cloned into the BamHI site (see "Materials and Methods"). Strains tested for growth on minimal medium pH 6.5 (left) and pH 8 (right) are: 1, pabaA1 argB2 araA49 chaA1 palB7; 2, pabaA1 argB2 araA49 chaA1 palB7::plJ16.1 (palB7 mutant strain transformed with plasmid plJ16.1, which contains the 6-kb BamHI restriction fragment shown above); 3, yA2 pabaA1 pyrG89::pUC19.181BamHI (palB gene disruption); 4, yA2 pabaA1 pyrG89 (recipient for palB gene disruption); 5, diploid yA2 pabaA1 pyrG89::pUC19.181BamHI/argB2 indB2 chaA1 palB7; and 6, diploid yA2 pabaA1 pyrG89::pUC19.181BamHI/chaA1 ahrA2 pantoA10.
mations were repeated with cosmids subpools until a single cosmid, 03H12, was identified. Transformants were confirmed as pal1 using additional phenotypic criteria (1,5). Subclones were tested by the same cotransformation procedure. palB cDNA clones were isolated by screening a lg10 library (10).

palB Gene Disruption—A 3.2-kb BglII restriction fragment containing the pyr4 gene of Neurospora crassa, which complements the pyrG89 mutation of A. nidulans, was cloned into the BamH I site of the plasmid pUC19.18, containing the SphI/KpnI fragment shown in Fig. 1B, to produce pUC19.18BamHI. This plasmid was linearized with SphI prior to transformation of a strain of genotype yA2 pabaA1 pyrG89.

Genomic DNAs of pyr1 transformants that were unable to grow on pH 8 medium were checked by Southern analysis for the predicted disruption restriction fragment pattern (data not shown). One verified disruption strain was used for meiotic analysis and to construct diploid strains for dominance and complementation analysis.

Sequence Analysis—The palB genomic sequence was determined on both strands by double-stranded sequencing of plasmids constructed in pUC19 using universal and gene-specific primers. The U.S. Biochemical Corp. Sequenase system was used. To identify introns, cDNA clones and PCR fragments generated by reverse transcriptase PCR were sequenced with the U.S. Biochemical Corp. system and with an automated sequencer.

Gel Mobility Shift Experiment—The gel mobility shift experiment was performed with growth conditions and mycelial extracts as described (4).

RESULTS

We cloned the palB gene using linkage group VIII clones from a chromosome-allocated, wild type A. nidulans cosmid library (8). To identify palB transformants, we used the inability of pal mutants to grow on alkaline pH media (1). The cosmid clone pWE15 03H12 rescued the palB7 strain to give a pal1 phenotype. This cosmid clone was shown by hybridization to overlap cosmid clones containing the ivoB gene, which is closely linked (2.1 centimorgans (11)) to palB (data not shown).

A 6-kb BamHI fragment of the cosmid was then found to rescue the palB7 strain (Fig. 1A). When this BamHI fragment was inserted into the argB1-containing plasmid pILJ16 and the resulting plasmid (pILJ16.1) used for transformation, all pal1 transformants analyzed had BamHI fragment-directed homologous integration (data not shown). One transformant, showing homologous integration, was crossed to an argB2 strain. All 104 progeny analyzed were palB1, and the argB1 and chaA1 markers showed 8% recombination, consistent with the linkage (12) between palB and chaA. This and the result that the larger, overlapping SphI restriction fragment rescued at a higher frequency suggested that the entire palB gene might not be contained within the BamHI fragment and that the BamHI site within the SphI fragment might be within the palB gene (Fig. 1). This BamHI site was therefore used for gene disruption (Fig. 1B). Strains disrupted at the BamHI site are unable to grow on pH 8 medium (Fig. 1B). Also, the re-
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Disruption mutation between tubA and pantoA in the expectation would therefore be that the BamHI site is within the gene.

Figure 2. This sequence spans the BamHI site within palB. The 3-kb XbaI/KpnI restriction fragment indicated by the striped bar in Fig. 1 and the 700-base-pair EcoRI/BamHI restriction fragment contained within it were used to probe Northern blots of wild type A. nidulans and to screen a cDNA library. Both probes hybridized to a message of approximately 3 kb. The boundaries of the largest cDNA clone isolated are shown by arrows in Fig. 2. We believe this cDNA clone contains all of the palB coding region for the following reasons. The clone contains a poly(A) tail, and all three reading frames upstream of the putative start codon are closed multiple times in the cDNA. Also, the size of this cDNA clone is close to our estimate of the mRNA size. This cDNA clone, a shorter cDNA clone, and reverse transcriptase PCR fragments were sequenced to determine the position of introns (Fig. 2). The resulting open reading frame would encode a protein of 842 amino acids and a molecular mass of 93.9 kDa. Sequence database searches with this open reading frame using the FASTA program (13) retrieve members of the calpain family of calcium-activated cysteine proteases. Strong sequence similarity between PalB and the calpains is limited to the catalytic region. A multiple sequence alignment of the catalytic region of several calpains and PalB is shown in Fig. 3. In addition to the Cys, His, and Asn residues believed to constitute the catalytic triad (14), starred in Fig. 3, PalB contains many conserved residues within the catalytic domain. In a pairwise comparison with human calpain p94, PalB has 29.1% amino acid identity over 300 amino acids and 32.3% identity over 189 amino acids of the catalytic domain. As indicated in Fig. 3, the BamHI site used for disruption separates the codon for the Cys catalytic residue from those for the His and Asn catalytic residues.

Activation of PacC in response to alkaline pH requires PacC proteolysis (4). A 29-kDa form of PacC, which is functional as a transcriptional activator and repressor, predominates at alkaline pH in wild type strains and irrespective of pH in pacC mutations (1, 3). It was necessary to determine experimentally whether processing of PacC to the 29-kDa form is affected by a putative EF-hand (17) overlined. The BamHI site used for gene disruption is indicated. The numbers refer to PalB residues. Calpains shown are: Cap1, human calpain (P07384); nCL-2, rat calpain (14479); p94, human calpain (P20807); Dros, D. melanogaster CalP (Z46891 and Z46892); Schis, Schistosoma mansoni calpain (P27730); Sol, D. melanogaster Sol protein (M64084). Accession numbers are given in parentheses. Sequence alignment was done with the GCG Pileup program (26).

Discussion

We have shown that a protein with sequence similarity to calpains is a component of the alkaline ambient pH signal transduction pathway in Aspergillus. Calpains contain C-terminal EF-hand structures which are responsible for calcium binding and regulation. PalB, like the Drosofila melanogaster Sol (small optic lobes protein) has similarity to the catalytic domain of calpains but not to these calcium-binding domains (15). However, PalB and Sol both have similarity to a putative EF-hand structure located immediately C-terminal to the catalytic domain (overlined in Fig. 3). Some putative EF-hand structures in known calpains deviate from the EF-hand consensus (16, 17). Allowing for such deviations, there are also several possible EF-hands in the C-terminal region of PalB. As hypothesized for the Sol protein (15), PalB might have
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substrate specificity similar to that of calpains but not be calcium-regulated. In vivo and in vitro substrates for calpains include cytoskeletal proteins, transcription factors, protein kinases, and protein phosphatases (18–23). Protein kinase C is proteolyzed to a permanently active form by calpain (19). Certain of these substrates are suggestive of signal transduction roles for calpains. Also suggestive of a calpain signal transduction role is the nuclear location of a muscle-specific calpain, which is mutated in one form of muscular dystrophy (24, 25).

PalB is required for growth at alkaline but not acidic pH. Although the palB mRNA is low in abundance, we have determined that there is little or no difference in its levels after growth in the pH range 4–8 (data not shown). The final proteolytic processing step for PacC is not catalyzed by PalB. The pal pathway is, however, responsible for a PacC modification that is required for its conversion to a functional form at alkaline pH (3, 4). The alkalinity-mimicking pacC–202 mutation removes 214 C-terminal residues from PacC (3). This deletion allows proteolytic processing of PacC to the 29-kDa functional form in the absence of the pal signal at acidic pH or in a pal mutant background (Fig. 4 and Ref. 4). The pacC–202 and other pacC mutation C-terminal truncations might destabilize intramolecular interactions, allowing proteolysis of PacC to the active form (4). In wild type strains, the pal pathway is thought to introduce a modification of PacC at alkaline pH, disrupting intramolecular interactions to allow activating proteolysis (4). The type of PacC modification mediated by the pal pathway in response to alkaline pH is not known, but an earlier (and more C-terminal) proteolytic cleavage of PacC resulting in susceptibility to further proteolysis might be mediated by PalB. Alternatively, PalB might proteolyze one of the other pal gene products in a signaling cascade. Sequencing of other pal genes and biochemical studies of PalB might help identify the PalB substrate(s).

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