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Trichophyton rubrum secreted and membrane-associated carboxypeptidases

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Abstract

Dermatophytes are the most common agents of superficial mycoses, and exclusively infect *stratum corneum*, nails or hair. Therefore, secreted proteolytic activity is considered a virulence trait of these fungi. In a medium containing protein as a sole nitrogen and carbon source *Trichophyton rubrum* secretes a metallocarboxypeptidase (TruMcpA) of the M14 family according to the MEROPS proteolytic enzyme database. TruMcpA is homologous to human pancreatic carboxypeptidase A, and is synthesized as a precursor in a preproprotein form. The propeptide is removed to generate the mature active enzyme alternatively by either one of two subtilisins which are concomitantly secreted by the fungus. In addition, *T. rubrum* was shown to possess two genes (*TruSCPA* and *TruSCPB*) encoding serine carboxypeptidases of the S10 family which are homologues of the previously characterized *Aspergillus* and *Penicillium* secreted acid carboxypeptidases. However, in contrast to the *Aspergillus* and *Penicillium* homologues, TruScpA and TruScpB enzymes are not secreted into the environment, but are membrane-associated with a glycosylphosphatidylinositol (GPI) anchor. During infection, *T. rubrum* secreted and GPI-anchored carboxypeptidases may contribute to fungal virulence by cooperating with previously characterized endoproteases and aminopeptidases in the degradation of compact keratinized tissues into assimilable amino acids and short peptides.

Keywords: Dermatophytes; *Trichophyton rubrum*; *Aspergillus fumigatus*; Carboxypeptidases

Introduction

Dermatophytes are human and animal pathogenic fungi which cause cutaneous infections (Kwong-Chung and Bennet, 1992; Weitzman and Summerbell, 1995). These fungi grow exclusively in the stratum corneum, nails or hair, and produce hydrolytic enzymes that degrade compact keratinized tissues. Like many other ascomycete fungi, dermatophytes secrete substantial proteolytic activity into a medium containing protein as a sole nitrogen and carbon source (Jousson et al., 2004a, b; Monod et al., 2005). Endoproteases and aminopeptidases secreted by dermatophytes show homology to those secreted by species of the genus *Aspergillus*. However, dermatophytes differ by secreting

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multiple endoproteases of the subtilisin subfamily (serine proteases S8A) and of the fungalysin family (metalloproteases M36) according to the MEROPS proteolytic enzyme database (http://merops.sanger.ac.uk) (Barrett et al., 2004). Specificity of individual endoproteases of the subtilisin family towards keratin substrate, and the multiplicity of these proteases reflect the high degree of specialization of dermatophytes (Jousson et al., 2004b). Secreted exoproteases so far characterized in *Trichophyton rubrum* include two leucine aminopeptidases (MEROPS M28), and two dipeptidyl-peptidases (MEROPS S9B and MEROPS S9C) (Monod et al., 2005).

The purpose of the present investigation was to further characterize the proteases secreted by dermatophytes. While several carboxypeptidases have been isolated from *Aspergillus* culture supernatants (Nakadai et al., 1972a, b; Svendsen and Dal Degan, 1998; Blinkovsky et al., 1999), there are no existing data for carboxypeptidase activity in dermatophytes, with the exception of a negative report (Danew et al., 1971). We show herein that *T. rubrum* secretes a metallocarboxypeptidase A (MEROPS M14A) at the same time as endoproteases and aminopeptidases in a protein medium. In addition, we demonstrate the presence of two serine membrane-associated carboxypeptidases (MEROPS S10), which were predicted to be glycosylphosphatidylinositol (GPI)-anchored according to the amino acid sequence of their gene translation products.

Materials and methods

Microbial strains and gene libraries

Trichophyton rubrum CHUV1673-05, *Arthroderma benhamiae* CBS112371 (Fumeaux et al., 2004) and clinical isolates of *Trichophyton mentagrophytes*, *Trichophyton soudanense*, *Trichophyton verrucosum*, *Trichophyton violaceum*, *Microsporum canis*, and *Microsporum gypseum* were used in this study. *Escherichia coli* LE392 was used for the propagation of the bacteriophage λEMBL3 (Promega, Madison, WI, USA). All plasmid subcloning experiments were performed in *E. coli* XL1 blue. *Pichia pastoris* GS115 and KM71 (Invitrogen, Carlsbad, CA, USA) were used for transformation and production of recombinant proteins. A λEMBL3 genomic library of *T. rubrum* and cDNA libraries of *T. rubrum* and *A. fumigatus* (Jousson et al., 2004a; Denikus et al., 2005) were previously constructed.

Proteases

Recombinant *T. rubrum* subtilisin 3 (TruSub3), subtilisin 4 (TruSub4), subtilisin 5 (TruSub5), subtilisin 7 (TruSub7), metalloprotease 1 (TruMep1), and metalloprotease 3 (TruMep3) were produced in our laboratory (Jousson et al., 2004b; unpublished results). Proteinase K, subtilisin Carlsberg, *Aspergillus oryzae* alkaline protease (protease XXIII), and trypsin were purchased from Sigma (St. Louis, MO, USA).

Dermatophyte growth media

All dermatophytes were routinely grown on Sabouraud agar and liquid medium (Bio-Rad, Hercules, CA, USA) or, to promote production of proteolytic activity, in soy protein liquid medium (SP) and keratin liquid medium (KSP) as previously described (Jousson et al., 2004a; Monod et al., 2005).

T. rubrum cDNA partial sequence database

Three thousand eight hundred and four clones of the *T. rubrum* cDNA library (Jousson et al., 2004a) were sequenced using an SP6 primer generating an EST collection. The sequencing was done by Synergene Biotech GmbH (Schlieren, Switzerland) on an ABI Prism 3100 DNA sequencer using BigDye Terminator chemistry (Applied Biosystems, Foster City, CA, USA). Pregap4 from Staden package version 1.5 was used to remove vector sequences, to evaluate sequence quality, and for conversion of data formats of the EST sequences. Filtered sequences were assembled by Gap version 4.10 in a database including 514 contigs and 1631 singletons for a total of 2145 clusters.

T. rubrum and *A. fumigatus* carboxypeptidase cDNAs

T. rubrum and *A. fumigatus* carboxypeptidase cDNAs were obtained by PCR with a standard protocol (Jousson et al., 2004a, b) using homologous sense and antisense primers (P1–P18, Tables 1 and 2) and 200 ng of DNA prepared from 10^6 clones of the cDNA libraries as a template.

Screening of the genomic library

Recombinant bacteriophage plaques (2×10^4) of a *T. rubrum* genomic DNA library (Jousson et al., 2004a) were immobilized on GeneScreen nylon membranes (Perkin-Elmer, Waltham, MA, USA). The filters were hybridized with ^{32}P-labeled DNA fragments under low-stringency conditions (Monod et al., 1994). The probes were amplified DNA of five *A. fumigatus* genes (*AfuCP1–AfuCP5*) which were obtained by PCR with the primers P15/P16, P17/P18, P19/P20, P21/P22, P23/P24, respectively (Table 1), and *A. fumigatus* genomic DNA as a template. Alternatively, the filters were