A yeast gene (BLH1) encodes a polypeptide with high homology to vertebrate bleomycin hydrolase, a family member of thiol proteinases

Ulla Magdolen a, Günter Müller b, Viktor Magdolen c and Wolfhard Bandlow a

a Institut für Genetik und Mikrobiologie, München (Germany), b c/o Hoechst Aktiengesellschaft Frankfurt am Main, Frankfurt (Germany) and c Max-Planck-Institut für Biochemie, Martinsried (Germany)

(Received 28 September 1992)

Key words: Bleomycin hydrolase gene; Antitumor drug; Cysteine proteinase; (S. cerevisiae)

We have purified bleomycin hydrolase from yeast (molecular mass 55000 Da). Using protein sequence-derived degenerate oligonucleotide primers and amplification by polymerase chain reaction, the yeast gene BLH1 was isolated and characterized. The deduced amino acid sequence (483 amino acids) exhibits surprisingly high homology to vertebrate bleomycin hydrolase (43% identical residues and 22% conserved exchanges). It contains three blocks of sequences found conserved in other members of the thiol proteinase family and thought to be associated with the catalytic centre. BLH1 is non-essential under all growth conditions tested. However, in the presence of 3.5 mg bleomycin/ml medium wild-type cells have a slight growth advantage compared to blhl mutant cells.

Bleomycin is a mixture of closely related glycopeptide antibiotics secreted from Streptomyces verticillus [1]. It causes radiomimetic DNA damage in bacteria and eukaryotes. It also inhibits growth of a number of squamous and small cell type tumors in situ and in vitro, although its exact mechanism of action is unknown. Due to its lack of hepatic, renal and bone marrow toxicities, the drug is extensively used in tumor therapy [1,2]. A more general use of the drug is, however, primarily hampered by two drawbacks, (i) the pulmonary toxicity [3] and (ii) the resistance of certain cell types and tissues to the drug due to high intracellular activities of the thiol proteinase bleomycin hydrolase [4–6]. The ubiquitous and abundant occurrence of this enzyme in all animal tissues and in microorganisms points to an important, although as yet unknown function. In order to study bleomycin resistance, to identify the physiological role of the proteinase and to understand why certain tumors are refractory to therapy by the drug, it may be helpful that we have isolated a yeast gene displaying high homology of the encoded protein to bleomycin hydrolase from rabbit.

We have isolated the bleomycin hydrolase protein from yeast during a study of a cAMP receptor glycoprotein-lipid-anchored to plasma membranes [7]. We have enriched the latter protein by isolating crude plasma membranes from yeast spheroplasts by Percoll gradient centrifugation and sequential purification of the detergent-solubilized protein by cAMP-agarose affinity chromatography, molecular sieving and phenyl Sepharose lipophilic chromatography which uses the hydrophobicity of the glycolipid anchor-containing protein as a parameter for separation. When the eluted proteins were separated on a sodium dodecylsulfate polyacrylamide gel, one of the major bands comigrated with the authentic, 8-N3-[32P]cAMP photoaffinity-labeled product (apparent molecular mass 54 kDa, as deduced from electrophoretic mobility) [7]. This material was excised from the gel, eluted and cleaved either with trypsin or CNBr. The peptides were separated by high performance liquid chromatography. Two major fragments (one from each cleavage) were sequenced (indicated in Fig. 2). It turned out from the sequence data (see below) that they obviously do not belong to the glycosyl-phosphatidylinositol-anchored cAMP-binding protein from yeast plasma membranes. Whether the enriched protein with homology to bleomycin hydrolase binds to cAMP agarose and phenyl Sepharose directly or interacts with the native cAMP receptor protein...
fortuitously or in a functional complex remains to be established in the future.

The corresponding gene was isolated by using the polymerase chain reaction method. Since the intramolecular arrangement of the two eluted and sequenced peptides was unknown, three primers were designed from each of them, two overlapping primers in the one and a third one in the reverse direction. Pairwise amplification by polymerase chain reaction and crosswise hybridization led to the identification of a DNA fragment, which encoded sequences from both protein fragments. The genomic DNA clone, pPMY 11.0, subsequently isolated from a gene bank constructed from BamHI-digested yeast DNA, contained about 11 kbp of genomic DNA and was characterized by restriction and hybridization analyses. The region cross-hybridizing to the amplified DNA fragment could be narrowed down to two HindIII fragments, which were sequenced subsequently from the central HindIII site in both directions to yield the complete DNA sequence of the yeast BLH1 gene (Fig. 1A, B). The nucleotide sequence analysis and the deduced primary structure of the protein are displayed in Fig. 2. The open reading frame comprises 483 triplets with a deduced molecular mass of the encoded protein of 55.5 Da, the calculated isoelectric point is at pH 9.58. Structural analyses by using the algorithms of Kyte and Doolittle, and Hopp and Woods do not reveal any striking structural features and suggest a globular, hydrophilic protein. Southern hybridization with a labeled DNA fragment, derived from the coding region of the BLH1 gene shows that BLH1 occurs in single copy in the yeast genome (one example is given in Fig. 1D, lane 1). The codon bias index [8] of 0.35 points to a relatively high expression. The DNA sequence flanking the gene at its 5′ side displays no obvious TATA box. However, many yeast genes are known which lack obvious TATA sequences. In the putative promoter region of BLH1 a perfect consensus sequence for an abundant DNA binding protein, Grf2p [9], is found, immediately followed by a pyrimidine-rich sequence (Fig. 2). Grf2p may have a role in nucleosome positioning by excluding nucleosomes from the vicinity of its binding site. The same combination of sequence motifs as in the BLH1-upstream region also occurs in the promoter region of the yeast profilin gene, PFY1 [10]. Deletion of these elements in the PFY1 promoter dramatically reduce transcription of PFY1 (H. Herrmann and W. Bandlow, unpublished results).

Data base searches using the Genetics Computer Group (GCG) Sequence Analysis Software Package [11] reveal significant sequence similarities to bleomycin hydrolase, a cysteine proteinase isolated from rabbit lung and liver. A cDNA fragment encoding the central portion of this enzyme has been obtained from rabbit liver and sequenced [6]. The alignment of the deduced rabbit and yeast protein sequences is shown in Fig. 3A. Within the region of the known rabbit sequence, the homology is highly significant (40 S.D. units, using the program ALIGN [12] with the mutation data matrix, 100 random runs and a penalty for a gap of 20). The yeast sequence extends beyond the known rabbit sequence by 21 amino acids to the N-terminus and 158 residues to the C-terminus. The overlap of the two proteins contains 43% identical amino acids and 22% conservative exchanges at comparable positions (Fig. 3A).
The search for protein motifs and domains reveals that a stretch of 17 amino acids (pos. 92–108 of Blhlp) is highly homologous to the region containing the cysteine residue conserved in the catalytic centre of eukaryotic thiol proteinases (Fig. 3B). These include calpain, plant and protozoan cysteine proteinases, vertebrate lysosomal and secretory cathepsins, and two developmentally co-regulated cysteine proteinases from the slime mold Dictyostelium discoideum. In addition, it can be seen from Fig. 3B that Blhlp from yeast also has significant homology to two sequence blocks of other cysteine proteinases (which were previously unknown from the rabbit enzyme) implied to contribute to the active centre, but conserved only in a subset of thiol proteinases.

In order to gain some information about the physiological role of the protein, we replaced the structural BLH1 gene with two different incomplete blhl alleles...
As detailed in Fig. 1C. For this purpose the coding sequence contained between the ClaI site at pos. +100 and the EcoRI site at pos. +615 was cloned into the yeast integration vector pRS306 harbouring the URA3 gene as a selective marker [13] to yield clone pPMP9-A. After cleavage of pPMP9-A with HindIII (pos. +383 of the coding region of BLH1) the resulting linear plasmid DNA was transformed into either haploid or diploid yeast wild-type cells. The correct integration of the plasmid by homologous recombination at the BLH1 locus leads to the duplication of those BLH1 DNA sequences cloned into pRS306, the resulting two incomplete copies being separated by sequences from the vector (Fig. 1C). The integration event was proven by Southern blotting of genomic DNA restricted with NdeI. The blot was probed with the BLH1-derived sequences cloned into pPMP9-A. DNA from wild-type cells yields a single signal corresponding to 1.1 kbp (Fig. 1D, lanes 1), whereas the haploid disruption diploids contain both, the wild-type and the disrupted allele. Consequently, all three signals can be seen with DNA from heterozygous diploid disruption strains (Fig. 1D, lanes 2 and 3) instead of 2.5 and 3.5 kbp (Fig. 1D, lanes 2 and 3) instead of complete copies being separated by sequences from the plasmid DNA. Hence, all three signals were observed with DNA from heterozygous diploid disruption strains (Fig. 1D, lanes 2 and 3) instead of 2.5 and 3.5 kbp (Fig. 1D, lanes 2 and 3) instead of complete copies being separated by sequences from the plasmid DNA. Consequently, all three signals can be seen with DNA from heterozygous diploid disruption strains (Fig. 1D, lanes 2 and 3). Mating of the haploid strain W303-1A and of the isogenic blhl mutant strains led to the formation of functional diploids, yet the spores germinated. It must be concluded from these results that the gene is dispensable for vegetative and meiotic growth, cell viability and differentiation. Finally, the haploid wild-type strain (with a functional BLH1 allele) was compared to the disruption strain with respect to growth in the presence of bleomycin. For this purpose co-cultivation experiments of the haploid strain W303-1A and of the isogenic blhl mutant strain were performed: cells of both strains pre-grown overnight in the presence of bleomycin were mixed and incubated for 2 days in the absence of 3.5 mg bleomycin/ml medium (which leads to about 50% growth inhibition). The ratio of the wild-type and mutant cells was monitored by replica-plating at the beginning of the experiment and after two days of growth by testing uracil auxotrophy (URA3 has been used as the selective marker to inactivate BLH1). The initial

**Fig. 3. Comparison of the deduced amino acid sequence of BLH1 to other cysteine proteinases:**

(A) Residues identical to the known sequence of rabbit bleomycin hydrolase [6] at corresponding positions are shaded. (B) Alignment of Bhlp with other cysteine proteinases in the regions containing the putative active-site cysteine, histidine and asparagine (indicated by stars) [14]. Amino acids identical to Bhlp are shaded. Sequences were taken from [6,14–19].
ratio of 55% blhl mutant and 45% wild-type cells changed after two days (i.e., 26 generations) to 27% mutant and 73% wild-type cells, indicating a slightly higher sensitivity of the mutant cells to the drug. In contrast, the ratios of both types of cells did not change significantly in the absence of bleomycin (55%/45% versus 56%/44% blhl /BLH1 cells). These results make it likely that BLH1, in fact, encodes a cysteine proteinase capable of hydrolyzing bleomycin. The lack of a detectable phenotype in blhl mutant cells in the absence of bleomycin as well as the only slightly increased sensitivity to bleomycin suggest that yeast may contain other proteinases with overlapping substrate specificities in addition to bleomycin hydrolase.

We thank E.-M. Wetekam for excellent technical assistance. The work was supported by a grant of the Deutsche Forschungsgemeinschaft to W.B. (Ba415/19-4).

References