Ligatoxin B, a new cytotoxic protein with a novel helix–turn–helix DNA-binding domain from the mistletoe *Phoradendron liga*

Shi-Sheng LI*, Joachim GULLBO†, Petra LINDHOLM*, Rolf LARSSON†, Eva THUNBERG*, Gunnar SAMUELSSON*, Lars BOHLIN* and Per CLAESON*¹

*Division of Pharmacognosy, Department of Medicinal Chemistry, Uppsala Biomedical Center, PO Box 574, SE-751 23, Uppsala, Sweden, and †Division of Clinical Pharmacology, University Hospital, Uppsala University, SE-751 85, Uppsala, Sweden

A new basic protein, designated ligatoxin B, containing 46 amino acid residues has been isolated from the mistletoe *Phoradendron liga* (Gill.) Eichl. (Viscaceae). The protein's primary structure, determined unambiguously using a combination of automated Edman degradation, trypsin enzymic digestion, and tandem MS analysis, was 1-KSCPSTTAR-NIYNTCRLTG-ASRSVCASLS-GCKIISGSTC-DSGWNH-46. Ligatoxin B exhibited *in vitro* cytotoxic activities on the human lymphoma cell line U-937-GTB and the primary multidrug-resistant renal adenocarcinoma cell line ACHN, with IC₅₀ values of 1.8 μ M and 3.2 μ M respectively. Sequence alignment with other thionins identified a new member of the class 3 thionins, ligatoxin B, which is similar to the earlier described ligatoxin A. As predicted by the method of homology modelling, ligatoxin B shares

a three-dimensional structure with the viscotoxins and purothionins and so may have the same mode of cytotoxic action. The novel similarities observed by structural comparison of the helix–turn–helix (HTH) motifs of the thionins, including ligatoxin B, and the HTH DNA-binding proteins, led us to propose the working hypothesis that thionins represent a new group of DNA-binding proteins. This working hypothesis could be useful in further dissecting the molecular mechanisms of thionin cytotoxicity and of thionin opposition to multidrug resistance, and useful in clarifying the physiological function of thionins in plants.

Key words: anticancer, homology modelling, mass spectrometry, polypeptide, thionin.

INTRODUCTION

Interest in extracts of mistletoes, semi-parasitic plants of the family Viscaceae which are most abundant in tropical areas [1], was initially pharmacological. Extracts of the European mistletoe (*Viscum album* L.) have been used for several decades against a variety of diseases, such as arthritis, rheumatism, and hypertension. They have also been used in the treatment of human epithelial tumours [2]. From *Viscum* extracts, different components with cytotoxic, immunoactive, and tumour-inhibiting properties have been identified, including the three type 2 ribosome-inactivating proteins, mistletoe lectins I–III, a chitin-binding agglutinin protein (visalbCBA), class 3 thionins (viscotoxins A1, A2, A3, B, 1-Ps), polysaccharides, oligosaccharides and alkaloids [3].

Recent research on mistletoe compounds has focused on the lectins and viscotoxin-like thionins of mistletoe, because of their encouraging antitumour activities [4,5]. Using a screening method for toxic proteins in plant extracts [6], viscotoxin-like proteins were also found in several other mistletoe species within the genera *Dendrophthora* [7], *Phoradendron* [8–11], and *Viscum* [6]. These small proteins contain 46 amino acid residues with three disulphide bridges, and have remarkable structural similarity. Evidence that these toxins resemble each other, not only in overall chemical structure, but also in biological and pharmacological behaviour, is growing [12]. Although their exact mode of action is still unclear, viscotoxins are present in the commercially available mistletoe preparations employed in alternative cancer

therapy [13]. Earlier, a mistletoe preparation was reported to be effective on cytostatic-drug-resistant human leukaemia cells *in vitro*, with drug-resistant sublines being even more sensitive to the preparation [14]. To tackle the increasing problem of cancer multidrug resistance (MDR), a recognized approach is to develop new anti-cancer drugs with multifaceted and unexplored mechanisms [15]. These observations prompted us to continue searching for new anti-cancer components of mistletoe origin. Using the fluorometric microculture cytotoxicity assay (FMCA) [16], we found that the protein fraction from *Phoradendron liga* was toxic to the human renal adenocarcinoma cell line ACHN, expressing primary MDR, and the sensitive lymphoma cell line U-937-GTB.

From *P. liga* growing on the host plant *Sapium longifolium* in Argentina, Ligatoxin A, a class 3 thionin [17], toxic to mammals when parenterally administered, was isolated and characterized [10,11]. From the same batch of a crude protein fraction, shown to be toxic to ACHN cells, a new basic protein containing 46 amino acid residues designated ligatoxin B, has been isolated and characterized. This paper describes the isolation, determination of primary structure and the cytotoxic activity of ligatoxin B and its sequence alignment with other thionins, and molecular modelling. Based on the extensive structure comparison of the helix–turn–helix (HTH) motifs of thionins and HTH DNA-binding proteins, we propose the working hypothesis that thionins constitute a new group of DNA-binding proteins. Later we propose a cytotoxic mechanism for ligatoxin B and a physiological role for thionins in plants.

Abbreviations used: CAM, carbamidomethyl cysteine; 3D, three-dimensional; ESI, electrospray ionization; FMCA, fluorometric microculture cytotoxicity assay; HTH, helix–turn–helix; i.d., internal diameter; MDR, multidrug resistance; MS/MS, tandem mass spectrometry; PDB, protein data bank; PEC, S-(β -4-pyridylethyl) cysteine; Q-TOF, quadrupole time-of-flight; RP, reverse-phase; SI, index for cell survival; TFA, trifluoroacetic acid.

¹ To whom correspondence should be addressed (e-mail per.claeson@mpa.se).

EXPERIMENTAL

Instruments and reagents

Semi-preparative, HPLC was performed using an ÄKTA basic HPLC system with UNICORN V3.10 software. UV absorption was monitored at 215, 254 and 280 nm, and an automatic Frac-901 collector was used. A Shimadzu LC-10 system equipped with an SPD-M10Avp photodiode-array detector was used for analytical HPLC; UV data was collected between 200 and 300 nm. For analytical and semi-preparative reverse-phase (RP)-HPLC, 250 mm × 4.6 mm (internal diameter; i.d.) and 250 mm × 10 mm (i.d.) Rainin Dynamax (C₁₈, 5 µm, 300 Å) columns were used respectively. CH₃CN, propan-2-ol and trifluoroacetic acid (TFA) were HPLC grade. A MilliQ water purification system (Millipore, Bedford, MA, U.S.A.) generated purified water. The sequence analysis was performed using an automated Edman degradation protein/peptide sequencer (model ABI 476A, Applied Biosystems/PerkinElmer). A nanospray-ion-trap-MS (Protana's NanoES source mounted on a Finnigan LCQ MS) was used to measure molecular mass. MS analysis was also conducted on a Micromass quadrupole time-of-flight (Q-TOF) electrospray instrument with a nanospray source (Manchester, U.K.). All mass spectra data were analysed in the positive mode using average isotopic masses for all calculated molecular masses. For bioassays, fluorescein diacetate (10 mg/ml) in DMSO was prepared, and the stock solution was kept under light-free conditions at -20 °C. Cell growth medium was prepared from RPMI 1640 stock supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM glutamine, 50 µg/ml streptomycin and 60 µg/ml penicillin. All chemicals used in the bioassays were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Plant material

Stems and leaves of *P. liga* (Gill.) Eichl. (Viscaceae) were collected from *Sapium longifolium* (Muell. Arg.) Huber (Euphorbiaceae) at Sta. Ana, Lacon (Argentina) by Dr Antonio Krapovickas (Facultad Agronomía y Veterinaria, Universidad Nacional del Nordeste, Corrientes, Argentina). The Division of Pharmacognosy (Uppsala University, Sweden) keeps a voucher specimen [10,11].

Extraction and isolation

A pooled protein fraction (designated the P fraction; 500 mg), obtained as described previously [10], and stored under light-free conditions at -20 °C, was used. In brief, the previous extraction and purification procedures comprised the following steps: (a) extraction of the plant material with 2% (v/v) acetic acid; (b) precipitation of mucilage by the addition of ethanol up to 46% (v/v), followed by centrifugation; (c) chromatography of the supernatant on carboxymethyl cellulose and Sephadex G-25 columns, successively (the desalted fraction was designated fraction A2); and (d) chromatography of fraction A2 on SP-Sephadex, which yielded three peaks including ligatoxin A (peak 3). The P fraction used in this study contained 9 mg of peak 1, 16 mg of peak 2 and 830 mg of fraction A2 (see Figure 1 in [10] pooled together).

The P fraction was subjected to semi-preparative RP-HPLC using a 250 mm × 10 mm [internal diameter (i.d.)] column, eluted with a programmed gradient from A to B over 68 min at a flow rate of 2.5 ml/min, where A was 23% organic modifier (CH₃CN/propan-2-ol, 6:4, v/v) and 0.1% TFA and B was 65% organic modifier (CH₃CN/propan-2-ol, 6:4, v/v) and 0.1% TFA (see Figure 1). Peak A was identified as ligatoxin A using amino acid analysis and MS as described previously (results not

Figure 1 HPLC profile of the P fraction

The 250 mm × 10 mm (i.d.) Rainin Dynamax (C₁₈, 5 µm, 300 Å) columns were used for the semi-preparative purification. Eluents: A, 23% organic modifier (CH₃CN/propan-2-ol, 6:4), 0.1% TFA; B, 65% organic modifier (CH₃CN/propan-2-ol, 6:4), 0.1% TFA. Flow rate: 2.5 ml/min. UV was monitored at 215, 254 and 280 nm. The profile with the absorbance at 280 nm versus retention time is shown here. Peak A was identified as the previously described protein, ligatoxin A, using amino acid analysis and MS (results not shown); Peak B, designated as ligatoxin B, was collected and purified further by analytical HPLC.

shown); peak B was collected for further purification. In the final purification by RP-HPLC, a 250 mm × 4.6 mm (i.d.) column was used, which was eluted with a linear gradient of 33–68% organic modifier (CH₃CN/propan-2-ol, 6:4, v/v) and 0.1% TFA over 60 min at a flow rate of 0.8 ml/min. A highly purified protein (50 µg), determined by quantitative amino acid analysis, was obtained.

Determination of primary structure

Quantitative amino acid analysis was conducted at the Amino Acid Analysis Center (Department of Biochemistry, Uppsala University, Sweden). In brief, the protein was hydrolysed for 24 h at 110 °C with 6 M HCl containing 2 mg/ml phenol; the hydrolysates were analysed with an amino acid analyser using ninhydrin detection.

For the sequence analysis, the protein was reduced with dithioerythritol in 0.25 M Tris/HCl containing 1 mM EDTA and 6 M guanidine/HCl (pH 8.5, 24 °C, 2 h). The reduced protein was subsequently alkylated to its *S*-(β-4-pyridylethyl) cysteine (PEC) derivative by adding 4-vinylpyridine to the solution (37 °C, 1 h). Reduction and alkylation were carried out in the dark under argon. The alkylated protein was then desalted and isolated using gel filtration on a Superdex Peptide HR 10/30 column (Amersham Biosciences, Uppsala, Sweden) in 40% CH₃CN in 0.1% aqueous TFA. The amino acid sequence was then determined with automated Edman degradation using a protein sequencer, according to the manufacturer's instructions.

For tandem mass spectrometry (MS/MS) analysis, the PEC derivative was subjected to tryptic cleavage (pH 8.1, 37 °C, 4 h) under an argon atmosphere. Another set of native proteins were reduced by incubation with 0.1 M Tris/HCl buffer (pH 8.5) containing 8 M guanidine/HCl, 10 mM EDTA and 20 mM dithiothreitol at 56 °C for 30 min. Cysteine residues were then alkylated with 20 mM iodoacetamide for 60 min at 20 °C (in

Table 1 Amino acid composition and molecular mass of ligatoxin B

Residues from amino acid analysis are listed on the left and residues from sequencing are listed on the right.

Amino acid	Ligatoxin B	
	Amino acid analysis	Sequencing
Asp/Asn (D/N)	4.2	3N*/1D
Thr (T)	4.9	5
Ser (S)	9.0	9
Pro (P)	1.4	1
Gly (G)	4.4	4
Ala (A)	3.0	3
Cys (C)	5.7†	6‡
Val (V)	1.1	1
Ile (I)	2.5	3
Leu (L)	2.1	2
Tyr (Y)	1.0	1
His (H)	0.9	1*
Lys (K)	1.8	2
Arg (R)	3.0	3
Trp (W)		1
Number of amino acids	46	
Molecular mass		
Calculated§	4792	
Measured	4792	

* Determined in combination with ESI-Q-TOF MS.

† Half-cystine was found to be cysteic acid with a separate sample following oxidation with performic acid.

‡ Cysteine, found to be PEC, following alkylation with 4-vinylpyridine.

§ Calculated using average masses with the total sum from amino acid composition adjusted to three disulphide bridges (−6 Da).

|| Determined by ion-trap Nanospray-ESI-MS.

darkness). Resulting peptides from digest and alkylation were separated and desalted by RP-HPLC. Before MS analysis, peptide fractions were pooled, dried in a Speedvac, re-dissolved in formic acid solution, and diluted with 50% acetonitrile in water. After the initial peptide scanning, individual peptides were selected for further fragmentation. Biolyx Peptide sequencing software aided the final analysis of the fragmentation pattern.

Alignment and molecular modelling

The amino acid sequences were aligned using the CLUSTAL W program [18]. The three consensus lines were generated by the separate alignments of mistletoe toxins, by the biologically active thionins, and by all the thionins. The gaps were manually modified in different groups to display conjointly the alignment results. The three-dimensional (3D) molecular modelling of the protein was carried out using the SWISS-MODEL program, with the first approach mode [19]. The Protein Data Bank (PDB) coordinate files of five templates were used to build the model: viscotoxin A3, PDB ID: 1ED0 [20]; crambin A, PDB ID: 1CCN [21]; crambin B, PDB ID: 1EJG [22]; α -l-purothionin, PDB ID: 2PLH [23]; and β -purothionin, PDB ID: 1BHP [24], with a high degree of sequence identity to the protein [25]. The 3D structures were visualized with the RasMol program (RasMac version 2.6) [26].

Cytotoxicity bioassays

The FMCA measures cell integrity based on fluorescence generated from hydrolysis of fluorescein diacetate to fluorescein

Table 2 MS/MS analysis of peptide sequences of the PEC tryptic peptides and the CAM derivative of ligatoxin B

Precursor ions	Peptide sequences*	Observed MS/MS ions†
PEC tryptic peptides		
568.3 ²⁺	2-SCCPSTTAR-10	296.1 (b2), 504.2 (b3), 632.3 (y6), 840.4 (y7), 1030.5 (y8-H ₂ O), 1135.5 (MH ⁺)
302.7 ²⁺	18-LTGASR-23	333.2 (y3), 390.2 (y4), 491.3 (y5), 604.3 (MH ⁺)
741.4 ²⁺	34-IISGSTCDSGWNH-46	86.1 (a1), 156.1 (y1), 199.2 (a2), 227.2 (b2), 270.1 (y2), 513.2 (y4), 600.2 (y5), 923.3 (y7), 1150.5 (y10-H ₂ O), 1168.4 (y10), 1255.5 (y11), 1368.6 (y12), 1481.7 (MH ⁺)
CAM parent ion		
1028.9 ⁵⁺	1-...-33-KIISGSTCDSGWNH-46	156.1 (y1), 253.1 (y2-NH3), 270.1 (y2), 513.2 (y4), 600.3 (y5), 715.3 (y6), 875.3 (y7), 976.4 (y8), 1063.4 (y9), 1120.4 (y10), 1207.5 (y11), 1320.6 (y12), 1433.6 (y13), 1561.7 (y14)

* L and I were derived from Edman sequencing, since these amino acids cannot be distinguished by MS/MS analysis.

† Nomenclature is according to [29,30].

by cells with intact plasma membranes. The substances, dissolved in 10% ethanol, were dispensed into microtitre plates (20 μ l/well). Two independent experiments were performed with triplicate samples. PBS and Triton X-100 (1%) were included as negative and positive controls respectively. Human tumour cells [27], from cell lines U-937-GTB (lymphoma) and ACHN (renal adenocarcinoma), were dispensed on the substance-charged microtitre plates and incubated at 37 °C with 5% CO₂ for 72 h without changing medium. The cells (2 $\times$ 10⁵/180 μ l) were then washed with PBS before adding fluorescein diacetate (10 μ g/ml) to each well. After incubating the plates for 40 min, the generated fluorescence was measured in a 96-well scanning fluorimeter (Fluoroscan II; Labsystems OY, Helsinki, Finland). The fluorescence, measured from a reaction well, is proportional to the number of living cells. The index for cell survival (SI) is the fluorescence of test wells, as a percentage of fluorescence for controls, with blank values subtracted. The IC₅₀ values, that is, the concentrations yielding an SI of 50%, were estimated from dose-response curves.

RESULTS

Isolation and characterization

The P fraction was subjected to repeated RP-HPLC (Figure 1), which yielded a homogeneous protein with an experimentally determined molecular mass of 4792 Da. In comparison with the calculated theoretical mass given by the quantitative amino acid and Edman sequencing, a difference of 6 Da was observed, which agrees with the six cysteines engaged in three disulphide bridges (Table 1). The protein, then reduced and alkylated with either 4-vinyl pyridine or iodoacetamide, yielded the PEC or carbamidomethyl cysteine (CAM) derivative. The experimentally determined mass data gave 5429 Da for the PEC derivative and 5141 Da for the CAM derivative, which again agrees with the theoretical mass data (giving 5429 Da or 5141 Da) for derivatives with all six cysteines alkylated by 4-vinylpyridine or iodoacetamide.

Figure 2 MS/MS spectrum of the C-terminal peptide from tryptic digest and the CAM derivative of ligatoxin B

L designates L or I, since these two amino acids cannot be distinguished by MS/MS analysis. (A) MS/MS spectrum of the tryptic C-terminal peptide ion 741.4^{2+} from ligatoxin B. Cysteines residues were alkylated with 4-vinylpyridine. The fragment ions matched the sequence 34-IISGTCDSGWNH-46. (B) The C-terminal MS/MS spectrum of the precursor ion 1028.9^{5+} of ligatoxin B. Cysteines were alkylated with iodoacetamide. The y series ions y1–y14 are consistent with the sequence 33-KIISGTCDSGWNH-46.

The N-terminal sequence was obtained by automated Edman degradation of the PEC derivative. Forty-six cycles were run, with the first 41 giving a clear, single-choice amino acid. The amino acid sequence, read manually, was 1-KSCPSTTAR-NIYNTCLTG-ASRSVCASLS-GCKIISGTC-D-41. G and W were assigned to cycles 43 and 44 as the first-choice amino acid; the rest of the cycles could not be read due to interfering background signals. Considering the measured molecular mass and the data from amino acid analysis, and considering that all mistletoe thionins reported so far contain 46 amino acid residues, positions 42, 45 and 46 are limited to being residues S, H and D/N.

To clarify the C-terminus further, the PEC derivative was digested with trypsin and sequenced by MS/MS using a Q-TOF mass spectrometer equipped with a nanoelectrospray ionization (nano-ESI) source [28]. Two doubly charged ions at m/z 568.3 and 302.7 were observed to correspond with the tryptic peptides 2–10 and 18–23, which was further confirmed by MS/MS sequence analyses of these ions (Table 2). A third doubly charged peptide appeared at m/z 741.4, which was inconsistent with all predicted internal peptides from the tryptic digest. This fragment, whose MS/MS data provided almost complete sequence information, as shown in Table 2 and Figure 2(A), was assumed to be the C-terminal peptide. The C-terminal peptide was thus identified as 34-IISGTCDSGWNH-46. Furthermore, direct fragmentation of the multiply charged parent ion of the CAM derivative at m/z 1028.9 also produced a continuous series of

predominant y-type ions (nomenclature according to [29] and [30]) from y1 up to y14, which led to the identical conclusion (Figure 2B). The highly accurate Q-TOF instrument resulted in unambiguous discrimination of N from D in position 45. Thus, the amino acid sequence of ligatoxin B (1-KSCPSTTAR-NIYNTCLTG-ASRSVCASLS-GCKIISGTC-DSGWNH-46) was unambiguously determined by the combination of N-terminal Edman sequencing and C-terminal MS/MS sequence analyses.

Amino acid sequence alignment

The designated protein consisted of 46 amino acids with one negatively charged residue (1D) and six positively charged residues (1H, 2K and 3R). Six cysteines in the thionin conserved positions suggested the presence of three disulphide bonds. These features identify ligatoxin B as a new member of the class 3 thionins [17].

Most of the thionins show toxic effects on bacteria, fungi, yeast and animal and plant cells, except for the class 4 thionins, i.e. the neutrally charged crambins from the seeds of *Crambe abyssinica* [31]. Therefore, the thionins isolated and characterized so far were grouped according to [17] as toxins of mistletoe origin (class 3), biologically active thionins (classes 1–3), and all the thionins (classes 1–4), and were aligned separately with ligatoxin B by means of the CLUSTAL W (1.81 version) program, as shown in Figure 3(A). In all mistletoe thionins (including

Ligatoxin B exhibited a high degree of sequence similarity with viscotoxin A3 (81 %), α 1-purothionin (54 %), β -purothionin (52 %), crambin B (48 %), and crambin A (44 %). The 3D structures of these thionins, shown to be compact molecules with a similar 3D core structure, have been studied extensively. The Greek capital letter gamma (Γ) has been used to denote this molecular conformation [33]. The vertical stem in Γ is a well-defined anti-parallel pair of α -helices with a highly flexible turn structure between them. The horizontal arm consists of two short

Figure 4 Stereoview of the 3D structures of ligatoxin B, viscotoxin A3 and A chain of the globular domain of histone H5

The view shows the localization and orientation of HTH motifs present; α -helices are magenta; β -sheets are yellow; turns are pale blue or white. R represents the recognition helix; R + 1 represents the first helix following the recognition helix; R + 2 represents the second helix following the recognition helix; R - 1 represents the first helix preceding the recognition helix; R - 2 represents the second helix preceding the recognition helix. (A) 3D structure of ligatoxin B from similarity molecular modelling using the SWISS-MODEL program. (B) 3D structure of viscotoxin A3. Its coordinate file was downloaded from the PDB (ID: 1ED0) [20]. (C) 3D structure of the A chain of the globular domain of histone H5. The coordinate file of its globular domain was downloaded from the (ID: IHST) [64], and only the A chain is visualized for comparison.

anti-parallel β -sheets and ill-defined C-terminal residues. In an attempt to predict the 3D structure of ligatoxin B, the five above-mentioned templates were used to model homology using the SWISS-MODEL program. Based on more than 50% sequence identity to templates, homology modelling can be highly accurate, comparable with medium-resolution NMR, and low-resolution X-ray crystallography [34].

The results depicted in Figures 3(B) and 4(A) indicate an overall homology of the 3D structure of ligatoxin B with that of other thionins. Ligatoxin B also has an amphipathic structure that derives primarily from the two amphipathic α -helices, which run in opposite directions; all charged groups and the several neutral polar side chains are at the inner bend between the helical stem and the β -arm, and are clearly separated from the hydrophobic underside and left side of the helical stem. The presence of three disulphide bridges, Cys³-Cys⁴⁰, Cys⁴-Cys³² and Cys¹⁶-Cys²⁶, stabilizes this 3D structure. Together with the turn structure at position 19-22, a HTH motif, characteristic of DNA-binding proteins, is predicted between residues 7 and 30 of ligatoxin B. The same feature has been observed in viscotoxin A3 [20].

Surprisingly, a third short α -helix was predicted at positions 42-44, where the hydrophobic residues Gly⁴³ and Trp⁴⁴ are mainly located at the backward surface of the β -arm, whereas the serine residue at position 42 is located at the front surface, which greatly improves the amphipathic character of the surface of ligatoxin B. The finding of a helix triplet in ligatoxin B corroborates its potential DNA-binding properties, since most of the currently known HTH DNA-binding domains comprise three helices [35].

A recent sequence analysis of the DNA-binding domain of the HTH motif-containing family of transcriptional regulators has shown that amino acid chemistry is conserved at many sites in the helices and the turn. A linear model was derived for identifying putative members of HTH family [36]. With this linear model, a comparison of the HTH motif in ligatoxin B and other members of the thionin family strongly suggests that the thionin HTH motif resembles the HTH motif in the DNA-binding domains of the transcriptional regulator family, but that the order of amino acids in the polypeptide chain is reversed (Figure 3B). Moreover,

Figure 5 Cytotoxic effects of ligatoxin B on the human tumour cell lines U-937-GTB (lymphoma) and ACHN (renal adenocarcinoma)

Values are means $\pm$ S.E.M. for two independent experiments run in triplicate. The IC_{50} values were graphically estimated from the concentration-response curves. In the ACHN cell line, the cells appear stimulated by ligatoxin B at concentrations below the IC_{50} value. This phenomenon has previously been observed with other DNA-interacting agents (Larsson, R., unpublished work).

the overall reverse-order homology of the 3D structures is remarkably high in the HTH motifs of the two families, exemplified by viscotoxin A3 and ligatoxin B, and by histone H5 respectively (Figure 4).

Cytotoxicity bioassays

Two cell lines, a sensitive cell line U-937-GTB and a primary MDR solid cell line ACHN [37], were used for screening and evaluating cytotoxic activities. The P fraction exhibited toxic activity with IC_{50} values of 42 μ g/ml on the U-937-GTB cells and 63 μ g/ml on the ACHN cells. Ligatoxin B, which was purified from the P fraction, expressed toxic activity with IC_{50} values of 8.4 μ g/ml (1.8 μ M) on the lymphoma cell line U-937-GTB and 15.3 μ g/ml (3.2 μ M) on the primary MDR renal adenocarcinoma cell line ACHN (Figure 5).

DISCUSSION

Ligatoxin B differs from the known toxic protein ligatoxin A (isolated from the same species) only at positions 21, 24 and 25

[10,11]. In the ligatoxins, one negatively charged (1D) and six positively charged residues (1H, 2K and 3R) are conserved, with the lysine and histidine residues as the N- and C-terminus. Six cysteines, also at conserved positions, form three disulphide bridges. Therefore, ligatoxin B is a new member of the class 3 thionins [17].

Ligatoxin B exhibited toxic activity on the tested human tumour cell lines U-937-GTB and ACHN. The latter is a primary MDR cell line, which was resistant to all available chemotherapeutic agents [37]. As a potential compound serving as a lead in searching for alternative cancer therapies, the toxic activity of ligatoxin B to ACHN cells is encouraging.

The alignment of biologically active thionins indicated that basic N- and C-termini, which are fundamental features for thionin biological activity, are mandatory for maintaining the toxicity of thionins. The 3D structure of ligatoxin B, predicted by homology modelling, shares overall similarity with such structures of viscotoxin A3, α 1-purothionin, β -purothionin and crambins, except for the third short amphipathic α -helix, predicted to be near the C-terminus of ligatoxin B. This interesting possibility, a helix triplet structure in ligatoxin B, strongly supports the idea that the thionins have intrinsic HTH DNA-binding properties, as discussed below.

Characteristics of HTH DNA-binding proteins

All HTH proteins share a site-specific DNA-binding domain composed of two α -helices separated by a short, variable-length turn. By means of this structural motif, the protein functions by binding along the major groove of the target DNA molecule. The first helix non-specifically associates with DNA, allowing correct positioning of the second helix. The second helix, commonly known as the recognition (or probe) helix, recognizes and binds to a specific consensus sequence along the major groove of the DNA molecule [38]. The turn motif typically contains three or four amino acids. Many members contain a third helix, which has a minor role in DNA binding, that of stabilizing the protein's hydrophobic core [35,39]. In general, the prokaryotic transcription factors bind to palindromic DNA sequences as homodimers, whereas eukaryotic proteins, such as members of the homeodomain family, bind both as monomers or heterodimers to non-symmetrical target sites [40].

By a series of phylogenetic analyses of the HTH motifs of transcriptional regulators, exemplified by the histones and bacterial repressors, a characteristic signature of the sequence has been summarized to identify HTH DNA-binding motifs [36]. Hydrophobic residues predominate at six of fourteen conserved sites. The only completely conserved sites are an alanine residue in the first helix and a glycine residue at the start of the turn. Four residues are constrained to being either basic or acidic residues, and two residues in the turn strongly favour a residue with a hydroxy moiety. All but two of the conserved positions reside in the first helix and the turn.

Novel HTH motif features of thionins

The comparison of amino acid sequences, as shown in Figure 3(B), revealed novel similarities between the HTH regions for thionins and for HTH DNA-binding proteins. In the thionins, the two basic residues in the first helix are conserved, whereas in the HTH motif of the DNA-binding proteins, the two conserved basic residues are located in the second helix. The conserved alanine residue and another conserved basic residue are located in the second helix of the thionins, and in the first helix of the HTH motif of DNA-binding proteins. Both have at the start of the turn the conserved glycine residue, which plays an

important role in maintaining the relatively dynamic orientation of the two helices. The 3D structures depicted in Figure 4 further disclose the overall reverse-order homology of the HTH motifs in the thionins and the transcriptional factors. Moreover, the HTH motif in plant thionins shares the similar turn structure with HTH DNA-binding proteins, such as the tryptophan aporepressor 3WRP, the λ repressors 2CRO, and 1LMB [41]. Considering protein–DNA interactions [39], these similarities strongly support our proposal that the thionins may have potential DNA-binding functions.

Experimental data suggest that some thionins, such as the cytotoxic viscotoxins, including viscotoxin A3, have DNA-binding properties *in vitro*, and that the viscotoxin A3–DNA interaction has some common features with the histone–DNA interaction for various histones [42]. Earlier, purothionin was reported to kill only cells in the DNA-synthesis phase [43], suggesting a possible specific interaction between purothionin and DNA molecules. Therefore, proposing the working hypothesis that the thionins represent a new group of HTH DNA-binding proteins seems reasonable.

Possible cytotoxic mode of ligatoxin B

Most of the basic thionins have been well known for being cell-membrane permeabilizing proteins *in vitro* that exhibit electrostatic interactions with negatively charged membrane phospholipids, followed by either pore-formation, or a specific interaction with a certain domain in the membrane [33]. β -Purothionin from *Triticum aestivum*, a homologue of ligatoxin B, forms cation-selective ion channels in artificial lipid-bilayer membranes and in the plasmalemma of rat hippocampal neurons [44]. Toxicological studies of *Pyrularia* thionin, also a homologue of ligatoxin B, show that the general response of cells to *Pyrularia* thionin involves alteration of the membrane leading to depolarization, a channel-mediated influx of Ca^{2+} , and to the related activation of phospholipase A_2 which results in the loss of membrane integrity or haemolysis, and eventually in cell death [45]. Viscotoxins, which are very similar to ligatoxins, were recently reported to cause accidental cell death and generation of reactive oxygen species in human lymphocytes, followed by secondary apoptosis-associated events, indicated by the expression of mitochondrial Apo2.7 molecules and caspase-3 activation [46,47]. These studies also demonstrated that the HTH motif and the amphipathic characteristics are important for maintaining the biological activities of the thionins.

Ligatoxin B has uncommonly high structural similarities with viscotoxins, β -purothionin, and *Pyrularia* thionin, not only at the amino acid level, but also in the modelled 3D structure, suggesting that all four compounds may have the same mode of action for cytotoxicity. Ligatoxin B, with its amphipathic features and proposed HTH-binding motif, may destroy the cell membranes of cancer cells by pore- (or ion)-channel formation. In addition, necrosis and/or apoptosis mechanisms may kill the 'surviving' cells, as observed in viscotoxin-treated human lymphocytes [46,47]. The presence of the proposed novel HTH DNA-binding domain in the thionins strongly favours the apoptosis mechanism. After entry into a 'surviving' cell, a thionin may cause direct DNA damage through its DNA-binding properties. DNA damage frequently triggers cell death by apoptotic pathways, as is well known [48].

The phoratoxins from *Phoradendron tomentosum* are the closest homologues to the ligatoxins among the thionins. A recent mechanism-based evaluation of cytotoxicities of these phoratoxins revealed that their activities were only slightly influenced by drug-resistance mechanisms mediated by P-glycoprotein,

topoisomerase II and GSH, although expression of a MRP-associated resistance mechanism was indeed observed (Johansson, S., Gullbo, J., Lindholm, P., Ek, B., Thunberg, E., Samuelsson, G., Larsson, R., Bohlin, L. and Claesson, P., unpublished work). Further searches in a database containing more than 100 antitumour agents disclosed that phoratoxins were most similar in drug-response patterns to digitoxin, a recently reported apoptosis-inducer [49]. Another preliminary report shows that viscotoxins are able to affect the intracellular *bcl-2* expression of human lymphocytes [50]. The over expression of anti-apoptotic genes, such as *bcl-2*, *bcl-x* and mutant *p53*, has been reported to be a new mechanism of acquired MDR for many cell lines, including U-937 [51]. These findings suggest that apoptotic pathways initiated by the DNA-binding properties of thionins may contribute to the observed opposition to the MDR phenomenon in tumour cells.

Possible physiological role for thionins in plants

In planta, thionins have been found to have a common biosynthetic pathway, starting with similar precursor structures, which consists of an N-terminal signal peptide, followed by the mature protein and a C-terminal acidic protein [33]. Thionins are predominantly located intracellularly, but are also found in the extracellular space [52]. Bohlmann and Apel [31] suggest that thionins may have evolved from a common ancestor, and play an important role in the growth and development of these plants. Previous studies indicate that high-level expression of thionin genes can enhance the resistance of transgenic plants to microbial attack [53], which strongly supports the hypothesis that thionins might play a role in plant protection against pathogens [54]. If bioactive thionins are released from the cell wall and the central vacuole, they may block the growth of the pathogen by functioning as plant extracellular defensins, with the known pore-forming action that destroys the cell membranes of attacking microbes.

During the pathogen invasion, one of the first defense reactions of plants is the hypersensitive reaction, a form of plant programmed cell death [55]. Cells close to the infection site collapse and form a necrotic area, which prevents spreading of a pathogen. So far, little is known about how this suicidal activity in plant cells is regulated. A recent report shows that the expression of thionin genes in *Arabidopsis thaliana* constitutively activated the octadecanoid pathway and formed necrotic microlesions [56]. The jasmonic acid-dependent octadecanoid pathway plays a major role in wound responses in *Arabidopsis*, and contributes to the control of some antifungal defence reactions [57]. Some of the thionin mutants even showed a strong capacity to induce both the salicylic acid- and the jasmonic acid-dependent signalling pathways [56]. For an intracellular function, thionins are likely to activate signalling networks that regulate innate responses, including the hypersensitive reaction in plants.

The proposed DNA-binding properties suggest that the intracellular thionins of an infected plant may actively participate in the onset of the transcription of related proteins. This leads to induction of necrosis and/or apoptosis of its own infected cells, thereby sacrificing those cells, which favours the survival of the plant. Within a wild plant population, a plant genotype with a mutant version of a thionin that improves the timing of induction of necrosis and/or apoptosis, with respect to disease resistance or suppression, will have superior fitness. Having superior fitness, the mutant thionin can have reasonably high probabilities of survival in the population, and then in time will replace existing inferior versions of the thionin. A logical and relevant next step in understanding the biological activities of thionins, and their

biochemical role in plant fitness within natural populations, is to investigate whether the physiological functions of those proteins are related to their proposed HTH DNA-binding properties.

Conclusions

A new basic protein, designated ligatoxin B, was isolated from *P. liga*, and was identified as a new member of class 3 thionins. Cytotoxicity tests showed that ligatoxin B was toxic to the lymphoma cells and the primary MDR renal adenocarcinoma cells studied. Ligatoxin B shares a 3D structure, predicted by homology modelling, with the viscotoxins and purothionins, and possibly shares the same mode of cytotoxic action. The newly observed structural similarities between the HTH motifs of the thionins, including ligatoxin B, and the HTH DNA-binding proteins led us to propose that the thionins may represent a new group of HTH DNA-binding proteins, which might be ubiquitous in the plant kingdom. Our proposal could help in dissecting the mechanisms of thionin cytotoxicity and thionin resistance to MDR phenomena, and thus further clarify their physiological function in plants.

We thank Dr Bo Ek (Department of Plant Biology, Swedish University of Agricultural Sciences, Uppsala) for assistance with the primary structure determination. We are very grateful to Dr Paul Johnson (AandZ Text Service, Uppsala) for critically reading the manuscript and helpful discussion.

REFERENCES

- Samuelsson, G. (1973) Mistletoe toxins. *System. Zool.* **22**, 566–569
- Stein, G. M., Schietzel, M. and Büssing, A. (1998) Mistletoe in immunology and the clinic. *Anticancer Res.* **18**, 3247–3250
- Zee-Cheng, R. K. Y. (1997) Anticancer research on Lorantheace plants. *Drug Future* **22**, 519–530
- Pae, H.-O., Seo, W.-G., Oh, G.-S., Shin, M.-K., Lee, H.-S., Lee, H. S., Kim, S. B. and Chung, H.-T. (2000) Potentiation of tumour necrosis factor- α -induced apoptosis by mistletoe lectin. *Immunopharmacol. Immunotoxicol.* **22**, 697–709
- Stein, G. M., Schaller, G., Pfüller, U., Schietzel, M. and Büssing, A. (1999) Thionins from *Viscum album* L.: influence of the viscotoxins on the activation of granulocytes. *Anticancer Res.* **19**, 1037–1042
- Samuelsson, G., Borsub, L., Jayawardene, A. L., Falk, L., Ziemilis, S. and Nilsson, Ö. (1981) Screening of plants of the families Lorantheace and Viscaceae for toxic proteins. *Acta Pharm. Suec.* **18**, 179–184
- Samuelsson, G. and Pettersson, B. (1977) Toxic proteins from the mistletoe *Dendrophthora clavata* II. The amino acid sequence of denclatoxin B. *Acta Pharm. Suec.* **14**, 245–254
- Mellstrand, S. T. and Samuelsson, G. (1974) Phoratoxin, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. *macrophyllum* (Lorantheaceae). The amino acid sequence. *Acta Pharm. Suec.* **11**, 347–360
- Thunberg, E. (1983) Phoratoxin B, a toxic protein from the mistletoe *Phoradendron tomentosum* subsp. *macrophyllum*. *Acta Pharm. Suec.* **20**, 115–122
- Thunberg, E. and Samuelsson, G. (1982) Isolation and properties of ligatoxin A, a toxic protein from the mistletoe *Phoradendron liga*. *Acta Pharm. Suec.* **19**, 285–292
- Thunberg, E. and Samuelsson, G. (1982) The amino acid sequence of ligatoxin A, from the mistletoe *Phoradendron liga*. *Acta Pharm. Suec.* **19**, 447–456
- Whitlow, M. and Teeter, M. M. (1985) Energy minimization for tertiary structure prediction of homologous proteins: α 1-purothionin and viscotoxin A3 models from crambin. *J. Biomol. Struct. Dyn.* **2**, 831–848
- Schaller, G., Urech, K. and Giannattasio, M. (1996) Cytotoxicity of different viscotoxins and extracts from the European subspecies of *Viscum album* L. *Phytother. Res.* **10**, 473–477
- Hülsem, H. and Mechelke, F. (1987) *In vitro* effectiveness of a mistletoe preparation on cytostatic-drug-resistant human leukemia cells. *Naturwissenschaften* **74**, 144–145
- Persidis, A. (1999) Cancer multidrug resistance. *Nature Biotechnol.* **17**, 94–95
- Larsson, R. and Nygren, P. (1989) A rapid fluorometric method for semi-automated determination of cytotoxicity and cellular proliferation of human tumor cell lines in microculture. *Anticancer Res.* **9**, 1111–1120.
- García-Olmedo, F., Carmona, M. J., Lopez-Fando, J. J., Fernandez, J. A., Castagnaro, A., Molina, A., Hernandez-Lucas, C. and Carbonero, P. (1992) Characterization and analysis of thionin genes. In *Genes Involved in Plant Defense* (Boller, T. and Meins, F., eds), pp. 283–302, Springer-Verlag, Vienna and New York

- 18 Thompson, J. D., Higgins, D. G. and Gibson, T. J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* **22**, 4673–4680
- 19 Guex, N., Diemand, A. and Peitsch, M. C. (1999) Protein modelling for all. *Trends Biochem. Sci.* **24**, 364–367
- 20 Romagnoli, S., Ugolini, R., Fogolari, F., Schaller, G., Urech, K., Giannattasio, M., Ragona, L. and Molinari, H. (2000) NMR structural determination of viscotoxin A3 from *Viscum album* L. *Biochem. J.* **350**, 569–577
- 21 Bonvin, A. M., Rullmann, J. A., Lamerichs, R. M., Boelens, R. and Kaptein, R. (1993) "Ensemble" iterative relaxation matrix approach: a new NMR refinement protocol applied to the solution structure of crambin. *Proteins* **15**, 385–400
- 22 Jelsch, C., Teeter, M. M., Lamzin, V., Pichon-Lesme, V., Blessing, B. and Lecomte, C. (2000) Accurate protein crystallography at ultra-high resolution: valence-electron distribution in crambin. *Proc. Natl. Acad. Sci. U.S.A.* **97**, 3171–3176
- 23 Rao, U., Stec, B. and Teeter, M. M. (1995) Refinement of purothionins reveals solute particles important for lattice formation and toxicity. Part 1: alpha-1-purothionin revisited. *Acta Crystallogr. Sect. D* **51**, 904–913
- 24 Stec, B., Rao, U. and Teeter, M. M. (1995) Refinement of purothionins reveals solute particles important for lattice formation and toxicity. Part 2: structure of beta-purothionin at 1.7 Ångströms resolution. *Acta Crystallogr. Sect. D* **51**, 914–924
- 25 Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., Bourne, P. E. and Peitsch, M. C. (2000) The Protein Data Bank. *Nucleic Acids Res.* **28**, 235–242
- 26 Sayle, R. A. and Milner-White, E. J. (1995) RASMOL: biomolecular graphics for all. *Trends Biochem. Sci.* **20**, 374–376
- 27 Dhar, S., Nygren, P., Csoka, K., Botling, J., Nilsson, K. and Larsson, R. (1996) Anti-cancer drug characterization using a human cell line panel representing defined type of drug resistance. *Br. J. Cancer* **74**, 888–896
- 28 Wilm, M., Shevchenko, A., Houthaave, T., Breit, S., Schweigerer, L., Fotsis, T. and Mann, M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature (London)* **379**, 466–468
- 29 Roepstorff, P. and Fohlman, J. (1984) Proposal for a common nomenclature for sequence ions in mass spectra of peptides. *Biomed. Mass Spectrom.* **11**, 601
- 30 Biemann, K. (1988) Contributions of mass spectrometry to peptide and protein structure. *Biomed. Environ. Mass Spectrom.* **16**, 99–111
- 31 Bohlmann, H. and Apel, K. (1991) Thionins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 227–240
- 32 Vernon, L. P., Evett, G. E., Zeikus, R. D. and Gray, W. R. (1985) A toxic thionin from *Pyricularia pubera*: purification, properties, and amino acid sequence. *Arch. Biochem. Biophys.* **238**, 18–29
- 33 Florack, D. E. A. and Stiekema, W. J. (1994) Thionins: properties, possible biological roles and mechanisms of action. *Plant Mol. Biol.* **26**, 25–37
- 34 Baker, D. and Sali, A. (2001) Protein structure prediction and structural genomics. *Science* **294**, 93–96
- 35 Wintjens, R. and Rooman, M. (1996) Structural classification of HTH DNA-binding domains and protein-DNA interaction modes. *J. Mol. Biol.* **262**, 294–313
- 36 Rosinski, J. A. and Atchley, W. R. (1999) Molecular evolution of helix-turn-helix proteins. *J. Mol. Evol.* **49**, 301–309
- 37 Okamoto, M., Hara, I., Miyake, H., Hara, S., Gotoha, A., Arakawa, A. and Kamidono, S. (2001) Synergistic anti-tumor effect of ionomycin and cisplatin against renal cell carcinoma *in vitro* and *in vivo*. *Urology* **57**, 188–192
- 38 Brennan, R. G. and Matthews, B. W. (1989) The helix-turn-helix DNA-binding motif. *J. Biol. Chem.* **264**, 1903–1906
- 39 Luscombe, N. M., Laskowski, R. A. and Thornton, J. M. (2001) Amino acid-base interactions: a three-dimensional analysis of protein-DNA interactions at an atomic level. *Nucleic Acids Res.* **29**, 2860–2874
- 40 Pabo, C. O. and Sauer, R. T. (1992) Transcription factors: structural families and principles of DNA recognition. *Annu. Rev. Biochem.* **61**, 1053–1095
- 41 Wintjens, R. T., Rooman, M. J. and Wodak, S. J. (1996) Automatic classification and analysis of $\alpha\alpha$ -turn motifs in proteins. *J. Mol. Biol.* **255**, 235–253
- 42 Woynarowski, J. M. and Konopa, J. (1980) Interaction between DNA and viscotoxins – cytotoxic basic polypeptides from *Viscum album* L. Hoppe-Seyler's Z. *Physiol. Chem.* **361**, 1535–1545
- 43 Nakanishi, T., Yoshizumi, H., Tahara, S., Hakura, A. and Toyoshima, K. (1979) Cytotoxicity of purothionin-A on various animal cells. *Gann* **70**, 323–326
- 44 Hughes, P., Dennis, E., Whitecross, M., Llewellyn, D. and Gage, P. (2000) The cytotoxic plant protein, β -purothionin, forms ion channels in lipid membranes. *J. Biol. Chem.* **275**, 823–827
- 45 Huang, W. H., Veron, L. P., Hansen, L. D. and Bell, J. D. (1997) Interactions of thionin from *Pyricularia pubera* with dipalmitoylphosphatidylglycerol large unilamellar vesicles. *Biochemistry* **36**, 2860–2866
- 46 Büssing, A., Stein, G. M., Wagner, M., Schaller, G., Pfüller, U. and Schietzel, M. (1999) Accidental cell death and generation of reactive oxygen intermediates in human lymphocytes induced by thionins from *Viscum album* L. *Eur. J. Biochem.* **262**, 79–87
- 47 Büssing, A., Vervecken, W., Wagner, M., Wagner, B., Pfüller, U. and Schietzel, M. (1999) Expression of mitochondrial Apo2.7 molecules and caspase-3 activation in human lymphocytes treated with the ribosome-inhibiting mistletoe lectins and the cell membrane permeabilizing viscotoxins. *Cytometry* **37**, 133–139
- 48 Rich, T., Allen, R. L. and Wyllie, A. H. (2000) Defying death after DNA damage. *Nature (London)* **407**, 777–783
- 49 Haux, J., Solheim, O., Isaksen, T. and Angelsen, A. (2000) Digitoxin, in non-toxic concentrations, inhibits proliferation and induces cell death in prostate cancer cell lines. *Z. Onkol.* **32**, 11–16
- 50 Büssing, A., Schaller, G. and Pfüller, U. (1998) Generation of reactive oxygen intermediates (ROI) by the thionins from *Viscum album* L. *Anticancer Res.* **18**, 4291–4296
- 51 Kojima, H., Endo, K., Moriyama, H., Tanaka, Y., Alnemri, E. S., Slapak, C. A., Teicher, B., Kufe, D. and Datta, R. (1998) Abrogation mitochondrial cytochrome c release and caspase-3 activation in acquired multidrug resistance. *J. Biol. Chem.* **273**, 16647–16650
- 52 Broekaert, W. F., Cammue, B. P. A., De Bolle, M. F. C., Thevissen, K., De Samblanx, G. W. and Osborn, R. W. (1997) Antimicrobial peptides from plants. *Crit. Rev. Plant Sci.* **16**, 297–323
- 53 Epplé, P., Apel, K. and Bohlmann, H. (1997) Overexpression of an endogenous thionin enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. *Plant Cell* **9**, 509–520
- 54 Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V. and Apel, K. (1988) Leaf-specific thionins of barley – a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defence mechanism of plants. *EMBO J.* **7**, 1559–1565
- 55 Heath, M. C. (1998) Apoptosis, programmed cell death and the hypersensitive response. *Eur. J. Plant Pathol.* **104**, 117–124
- 56 Hilpert, B., Bohlmann, H., Camp, R. O. D., Przybyla, D., Miersch, O., Buchala, A. and Apel, K. (2001) Isolation and characterization of signal transduction mutants of *Arabidopsis thaliana* that constitutively activate the octadecanoid pathway and from necrotic microlesions. *Plant J.* **26**, 435–446
- 57 Farmer, E. E., Weber, H. and Vollenweider, S. (1998) Fatty acid signaling in *Arabidopsis*. *Planta* **206**, 167–174
- 58 Bohlmann, H. and Apel, K. (1987) Isolation and characterization of cDNAs coding for leaf-specific thionins closely related to the endosperm-specific hordothionin of barley (*Hordeum vulgare* L.). *Mol. Gen. Genet.* **207**, 446–454
- 59 Bekes, F. and Laszity, R. (1981) Isolation and determination of amino acid sequence of avenothionin. A new purothionin analogue from oat. *Cereal Chem.* **58**, 360–361
- 60 Bekes, F., Smied, I. and Laszity, R. (1982) Investigation of thionins of wheat, barley, rye and oat. *Devel. Food Sci.* **5**, 163–168
- 61 Hernandez-Lucas, C., Royo, J., Paz-Ares, J., Ponz, F., Garcia-Olmedo, F. and Carbonero, P. (1986) Polyadenylation site heterogeneity in mRNA encoding the precursor of the barley toxin α -hordothionin. *FEBS Lett.* **200**, 103–105
- 62 Jones, B. L. and Mak, A. S. (1976) Amino acid sequences of the two α -purothionins of hexaploid wheat. *Cereal Chem.* **54**, 511–523
- 63 Taylor, W. T. (1986) The classification of amino acid conservation. *J. Theor. Biol.* **119**, 205–218
- 64 Ramakrishnan, V., Finch, J. T., Graziano, V., Lee, P. L. and Sweet, R. M. (1993) Crystal structure of globular domain of histone H5 and its implications for nucleosome binding. *Nature (London)* **362**, 219–223

Received 4 February 2002/22 May 2002; accepted 6 June 2002

Published as BJ Immediate Publication 6 June 2002, DOI 10.1042/BJ20020221