

A strategy for the identification of T-cell epitopes on *Leishmania* cysteine proteinases

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Key words: multiple sequence alignment, human leucocyte-associated antigen, cysteine proteinases, *Leishmania*, T-cell epitope

Abstract

In this study computational analysis was used to compile sequence alignments, construct a dendrogram and calculate physical data in order to predict potential T-cell epitopes of the *Leishmania* cysteine proteinase. Using multiple alignment of human and *Leishmania* proteinase sequences deposited on data bank sequences, it was possible to predict that the extreme C-terminus of cysteine proteinase (Cyspep, 355-444) contained three peptides (pI 361-370, pII 415-422 and pIII 431-444) with charge score, hydrophobicity and isoelectric points compatible for human leucocyte-associated antigen (HLA) class II binding. The prediction was confirmed *in vitro* through the ability of synthetic peptides corresponding to the predicted regions to stimulate peripheral blood mononuclear cells of patients with leishmaniasis.

Introduction

Leishmania are obligate intracellular protozoan parasites responsible for a large spectrum of clinical manifestations in man, including self-healing cutaneous, diffuse cutaneous and mutilating mucosal lesions, as well as lethal visceral disease (Ridley, 1987). The parasites are dimorphic, existing as flagellate extracellular forms (promastigotes) in the sandfly vector, and as intracellular aflagellate forms (amastigotes), in the mammalian host.

The protective immunity against *Leishmania* is mediated by T-cells (Convit *et al.*, 1972; Howard *et al.*, 1980; Mitchell *et al.*, 1981; Coutinho *et al.*, 1996). Thus, the identification of linear motifs of the parasite proteins which can be processed by antigen-presenting cells associated with human leucocyte-associated antigen (HLA) class-II molecules to CD4⁺ T-lymphocytes, would be of great relevance for designing studies aiming at inducing immunity against *Leishmania* (Liew and O'Donnell, 1993).

Structural motifs in proteins of the cysteine proteinase family are particularly potent epitopes for T-cell or antibody responses (Urban *et al.*, 1992; McKerrow, 1993). Cysteine proteinases have been identified and characterized in several species of *Leishmania* (Pupkis *et al.*, 1986; Lockwood *et al.*, 1987; Robertson and Coombs,

1990; Balanco *et al.*, 1991; Alves *et al.*, 1993; Alves *et al.*, 1998; Traub-Cseko *et al.*, 1993). However, the potential of *Leishmania* cysteine proteinases for inducing T-cell response has not been ascertained.

Algorithms for the identification of protein sequences which may bind to major histocompatibility complex (MHC) molecules have been proposed (Rothbard and Taylor, 1988; Cornette *et al.*, 1989). In the present study, potential T-cell epitopes on the COOH-terminus region of *Leishmania* cysteine proteinases were identified using (1) multiple sequence-alignment to compare human and *Leishmania* cysteine proteinases, (2) structural motif analysis and (3) physical datum determination.

Materials and methods

Protein sequences

Database searches for identical or homologous amino acid sequences were performed, using the software package of the Genetics Computer Group (GCG; Wisconsin University, Madison, Wisconsin, U.S.A.), version 7-UNIX. Most HLA class II and cysteine proteinase sequences were obtained from the PIR (Protein Identification Resource, National Biochemical Research Foundation, U.S.A.) and GenBank National Center for Biotechnology Information, U.S.A.). In the latter case only the coding sequence was used in the analyses.

Sequence alignment and motif analysis

Homology relationships between proteins was assessed using the GAP algorithm (Smith and Waterman, 1981). The multiple-sequence analysis (MSA) was undertaken using the PILEUP algorithm (Feng and Doolittle, 1987) and the PEPLOT to predicted curves for secondary structures [α -helix and β -sheet, hydrophathy, hydrophilicity according to Chou and Fasman (1974) and Kyte and Doolittle (1982)]. The physical properties of the amino acid sequences, molar extinction coefficient, the isoelectric points and molecular weights were calculated using PEPTIDESORT algorithms.

Synthetic peptides

All sequences were assembled manually using N-fluorenylmethoxy-carbonyl (Fmoc)-protected amino acids (Novabiochem, L aufelfingen, Switzerland) (Ferreira da Cruz *et al.*, 1996). The purity of the three peptides, as determined by analytical reverse phase high performance liquid chromatography (HPLC; C18-column; water/acetonitrile/trifluoroacetic acid, gradient from 99:1.0:0.1 to 30:70:0.1), was <70%.

Small aliquots of peptides were purified by semi-preparative HPLC and subjected to a sequencer protein (PSQ-1, Shimadzu, Kyoto, Japan). The purified peptides were deposited on TFA-polybrene treated glass filters and the sequence conducted as described previously (Giovanni De Simone *et al.*, 1994). The peptides were dissolved (1 mg ml^{-1}) in (PBS; pIII) or (DMSO; pI and pII) for the proliferation assay.

Peripheral blood mononuclear cells (PBMC) cultures

The PBMC, obtained from ten infected and ten uninfected individuals, were incubated for 5 days at 37°C in 5% CO_2 with Roswell Park Memorial Institute (RPMI) 1640 medium. The latter supplemented with 10% human AB serum contained 0, 3, 10 or $30 \mu\text{g ml}^{-1}$ of the different peptides, *Leishmania braziliensis* lysate ($30 \mu\text{g ml}^{-1}$) or $1 \mu\text{g ml}^{-1}$ of concanavalin A (Con A) in triplicate. The proliferative response was assessed by measuring the incorporation of radiolabelled thymidine (DeLuca *et al.*, 1999) and expressed as the stimulation index calculated from mean counts per minute (cpm) of experimental cultures divided by the mean of the control cpm cultures, *i.e.* cultures without antigen or Con A.

Results and discussion

The dendrogram generated by PILEUP algorithm (Figure 1) shows one principal cluster containing Lpcys1, Lpcys2, Lmacpa, Lmecpb, Lmecpc, Ldccys1, Ldccys2, CatL and CatS, and another cluster disclosing a relationship among Lmecpa, Lmacpb and CatB. A similarity graph plotted with sequence data from all molecules studied revealed that the COOH-terminal regions in the alignment had scores close to zero in regard to similarity, whereas most of the remaining sections had varying degrees of similarity (Figure 2). Indeed, sequences showed that the COOH-terminal regions of *Leishmania* cysteine proteinases did not relate to the primary sequence of our human cysteine proteinase studies (not shown). Therefore, it was likely that the COOH-terminal sequence (355-444) of *Leishmania* cysteine proteinases possessed appropriate epitopes for human T-cell activation, more than the other regions. Indeed, since it did not cross-react with their human proteinase counterparts, there was no immunological tolerance to them. This sequence had three stretches with a hydrophobic score above 0.6 (Figure 3). The peptides corresponding to these stretch sequences were synthesized and tested as T-cell epitopes: *viz* pI (361-370), pII (416-423) and pIII (431-444).

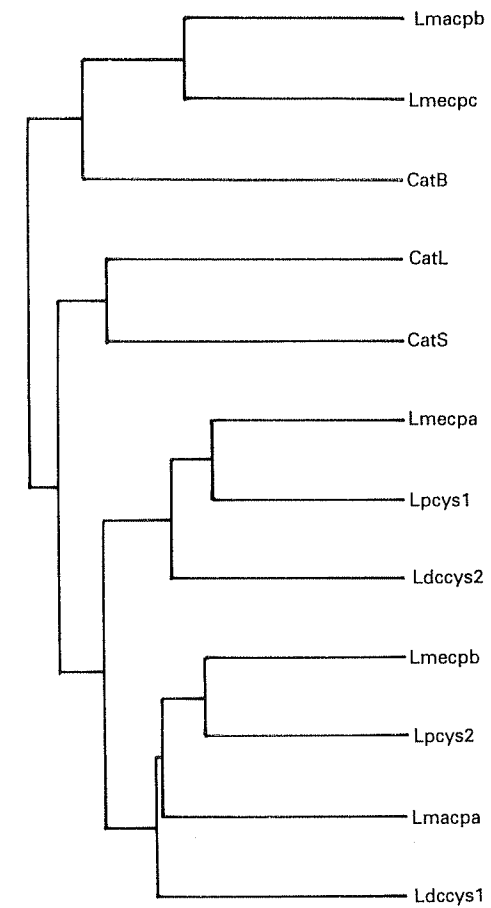


Figure 1 Structural dendrogram profile of proteins of the cysteine-proteinase family. The dendrogram was built with PILEUP algorithms based on the amino acid sequences of human cathepsin L (CatL; f27011), cathepsin B (CatB; a25432) and cathepsin S (Cat S; m86553) and *L. pifanoi* Lpcys1 (b48566) and Lpcys2 (q05094), *L. amazoensis* Lmecpa (u43705) and Lmecpb (p36400) and Lmecpc (z48599), *L. major* Lmacpa (p25775) and Lmacpb (u43706), *L. donovani* Ldccys1 (af004592) and *L. chagasi* Ldccys2 (af004593). The closeness of branches represents the degree of homology between different molecules.

It has been demonstrated that hydrophobic residues are required for the interactions of peptides with a conserved hydrophobic pocket in HLA class II molecules (Hill *et al.*, 1991; Brown *et al.*, 1993). The chosen sequences may have a β -sheet (pI and pIII) or an α -helix (pII) configuration (Figure 3). The secondary structure of the peptides may well play a role in determining the extent to which individual amino acids are exposed to interactions with sites within the MHC pocket.

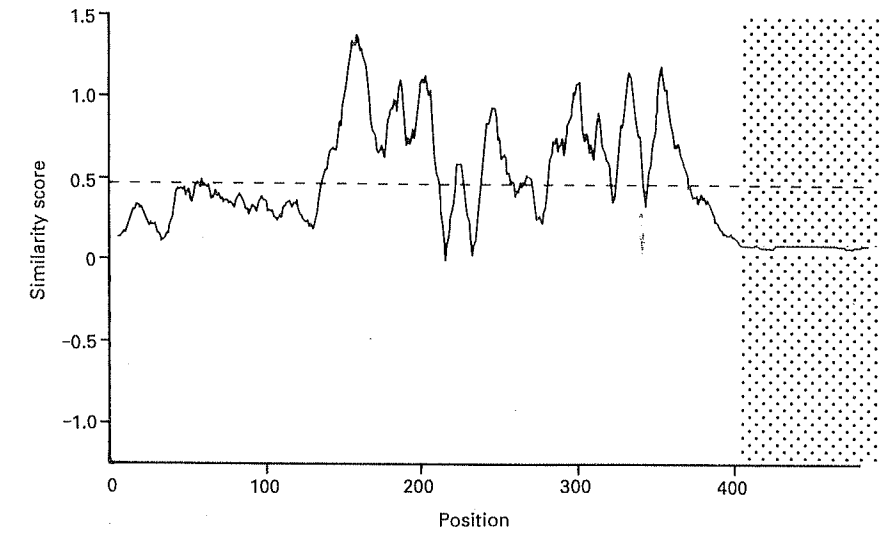


Figure 2 Average similarity amongst all sequences. The similarity score was calculated from PILEUP algorithms (Feng and Doolittle, 1987; Higgins and Sharp, 1989), based on the amino acid sequence of human and *Leishmania* cysteine proteinases, using the PLOTSIMILARITY algorithm (Smith and Waterman, 1981). Shaded areas indicate the amino acid positions with lower similarity scores.

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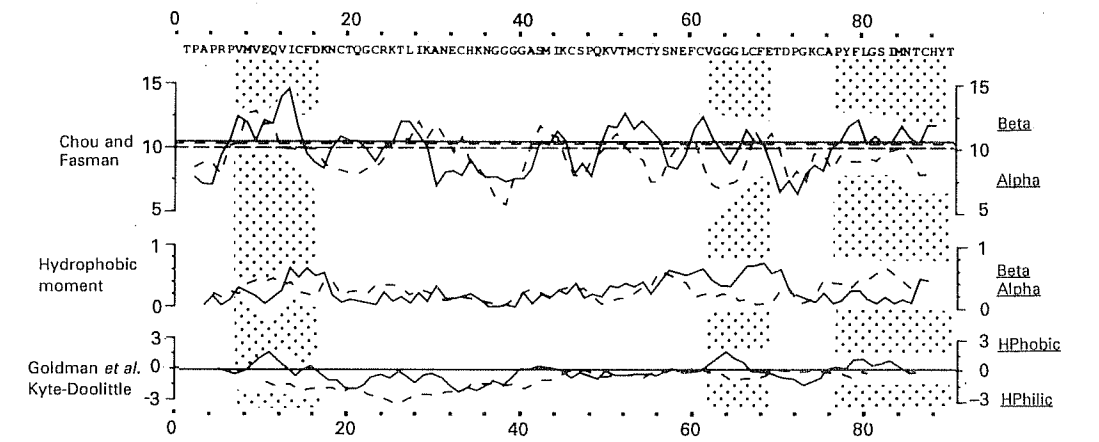


Figure 3 Hydrophobic, hydrophilic and secondary structure profile of Cyspep. The peptide sequence was predicted by exclusion analysis of human cysteine proteinase sequence hydrophobicity and hydrophilicity values were calculated according to Kyte and Doolittle (1982). For plotting α -helix and β -sheet structures, the method of Chou and Fasman (1978) was used. The shaded regions identify the sequences of the selected peptides with hydrophobic scores of >0.6 : pI (VMVEQVLCFD), pII (VGGGLCFE) and pIII (PYFLGSIMNTCHYT).

Determination of conformational and structural characteristics of the peptides binding to HLA molecules is crucial for defining potential T-cell epitopes. The algorithms used in this study helped us to determine which peptides possessed the structural features which may be important for the binding of small peptides from *Leishmania* cysteine proteinases to HLA molecules. The selected peptides pI, pII and pIII, from the cyspep sequence, have highly hydrophobic scores which is a significant feature of binding, comparable with peptides which bind to HLA-DR1Dw1 molecules (Jardetzky *et al.*, 1990; Hill *et al.*, 1991).

Charged residues in both HLA class II and peptide sequences are also important for binding. The charge, calculated by PEPTIDESORT algorithm, shows a motif containing a negatively charged residue in pI and pII (data not shown), which may bind to the positively charged HLA class II molecules: DPw4b, DQb, DQw9 and DRw4 alleles (Boehncke *et al.*, 1993).

The isoelectric points (IP) of the predicted epitopes, calculated by PEPTIDESORT algorithm, were above the processing and the binding pH. The IP were calculated from the amino acid sequence alone and do not take into account the changes in pKa of the side groups in response to tertiary environment. Moreover, it did not account for glycosylation. It was observed that pI, pII and pIII had IP of 3.58, 3.90 and 7.29, respectively, while the IP of fifteen different HLA sequences, calculated in this work, varied from 4.4 to 11.26 (data not shown). The IP may be a crucial factor for the binding of peptides to MHC, since it may depend on peptide solubility. In accordance with this view, all HLA molecules examined in this paper, although having large IP, were soluble at the endocytic pathway pH. The importance of pH for peptide-MHC interactions was further supported by observations on quantitative loading of peptides to class II in low pH conditions (Sadegh-Nasseri and Germain, 1991), and by the fact that the binding of some peptides was accelerated by the low pH of endosomal compartments (Jensen, 1990; Harding *et al.*, 1991; Reay *et al.*, 1992).

The T-cell antigenicity of the peptides was determined by testing their stimulatory effect on lymphocytes from patients with active cutaneous leishmaniasis, *in vitro*. Diagnosis was confirmed by visualization of *Leishmania* amastigotes in histopathological examination. The mean cpm in control cultures from normal donors \pm standard deviation was 595 ± 30 , and in the control cultures from infected donors was 280 ± 50 . The proliferative responses to Con A, were intense and comparable in patients and normal donors (mean stimulation index (SI) \pm standard deviation = 64 ± 25). The lympho-

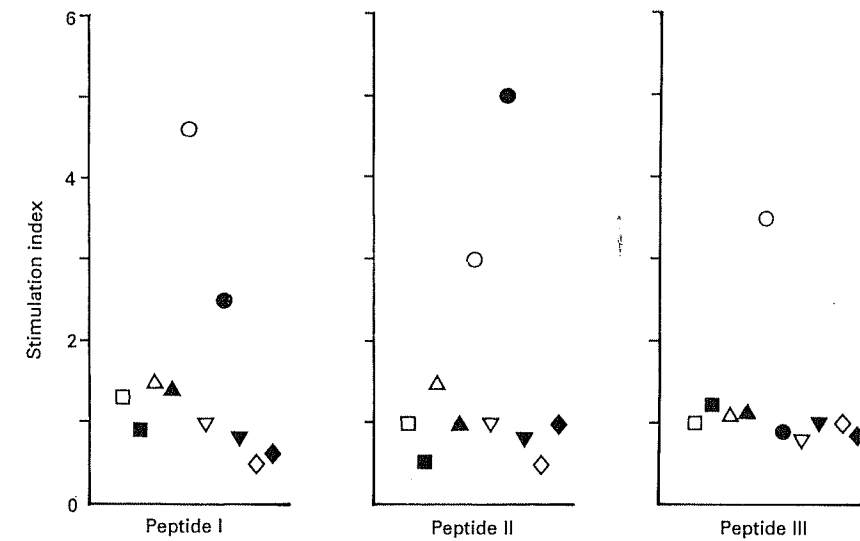


Figure 4 Lymphoproliferative responses of the *Leishmania*-infected individuals to synthetic peptides. All patients analysed in this study had a skin lesion from which *Leishmania* parasites were isolated. Mononuclear cells were isolated from whole blood by density centrifugation and resuspended in appropriated medium for incubation with peptides ($30 \mu\text{g ml}^{-1}$) or crude *L. braziliensis* lysate ($30 \mu\text{g ml}^{-1}$) for 5 days at 37°C . The wells were pulsed with $1 \mu\text{Ci}$ of $[^3\text{H}]$ -thymidine for the final 16 h of culture. The radioactivity uptake by the cells was measured in a scintillation beta counter and the lymphoproliferative activity was represented by the stimulation index. Symbols represent means of triplicate wells for each patient analysed in the study.

proliferative response to *Leishmania braziliensis* antigen was positive (SI >2.5 for $30 \mu\text{g ml}^{-1}$ of antigen) in three out of the ten patients tested (Figure 4). Comparable proliferative responses (SI >2.5) were observed, using the maximal peptide concentration ($30 \mu\text{g ml}^{-1}$) with peripheral blood mononuclear cells (PBMC) from two patients (Figure 4).

Our results demonstrate the feasibility of the proposed strategy for the identification of antigenic peptides and, as previously discussed, indicated that no single peptide will be immunogenic for all individuals. In consequence a peptide vaccine aimed at protecting a population against a micro-organism would need to be composed of several peptides.

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Research note

Mapping of the N terminus of the *Schistosoma mansoni* tegumental antigen Sm15 to its predicted precursor protein

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Abstract

Sm15 is a major *Schistosoma mansoni* 15 kDa tegumental antigen, resulting from the proteolytic processing of a larger precursor. The amino terminus of Sm15 was identified by direct amino acid sequencing, and the antigen was tentatively mapped to the segment spanning amino acids 362–497 of the precursor. This will allow subsequent studies to elucidate the possible immunological role of proteolytic processing in schistosomiasis. © 2000 Australian Society for Parasitology Inc. Published by Elsevier Science Ltd. All rights reserved.

Keywords: *Schistosoma mansoni*; Tegumental antigen; Sm15; Precursor protein; Purification; Mapping

As previously demonstrated by antibodies from mice protectively vaccinated with adult worm tegumental membranes [1,2], Sm15 is a partially characterised major 15 kDa tegumental antigen present in 2 to 6-week-old *Schistosoma mansoni* worms [2,3]. The gene for Sm15 encodes a much longer protein than that identified in tegumental membranes, indicating that it encodes a precursor that is subsequently highly processed [2,3]. In addition, there is evidence that the precursor is differentially processed during maturation, providing the parasite with the capability of expressing different products of the gene at different life cycle stages [2]. In order to further characterize the biosynthesis of Sm15 and allow subsequent studies to elucidate the possible immunological role of proteolytic processing in schistosomiasis, we report the mapping of Sm15 to its precursor.

Although the precise coding region of Sm15 was unknown, previous work had shown that some of the epitopes were present between amino acids 692–1032 (λ gt11 A70 cDNA) [3]. Thus, constructs overlapping this cDNA, were subcloned in pGEXTM, expressed in *Escherichia coli* as GST-(glutathione-S-transferase)-fusion proteins, and analyzed

by immunoblotting with monospecific antibodies to Sm15 (A70 R α M). These antibodies were obtained by affinity purifying anti-adult tegumental membrane antiserum by adsorption against the λ gt11 A70 cDNA expressing a β -galactosidase-fusion protein [2]. In addition to recognizing native Sm15, these antibodies recognized specifically the following GST-fusion proteins: 2TR7A (corresponding to the amino acids 444–788 of the Sm15 precursor), A70H1G2 (amino acids 692–858) and A70-20 (amino acids 692–1032) (Figs. 1 and 2). Fig. 2 shows a region of repeats previously described in detail [3], and the relationship of the fusion proteins with this region. These results suggested that at least some major epitopes of Sm15 are located between amino acids 692–788, which represents the region of overlap of the three fusion proteins. Further work involved the purification and partial amino acid sequencing of native Sm15. The protein was isolated from adult tegumental membrane proteins obtained essentially according to the freezing-thawing method [4]. The preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblotting, and the band, identified as Sm15 by rabbit anti-adult tegumental serum, was sequenced [5]. Partial sequencing resulted in the six amino acid sequence GTVGEM (with some uncertainty respecting the 6th residue), which maps to amino acids 362–367 of the sequence of the Sm15 precursor (Fig. 2),

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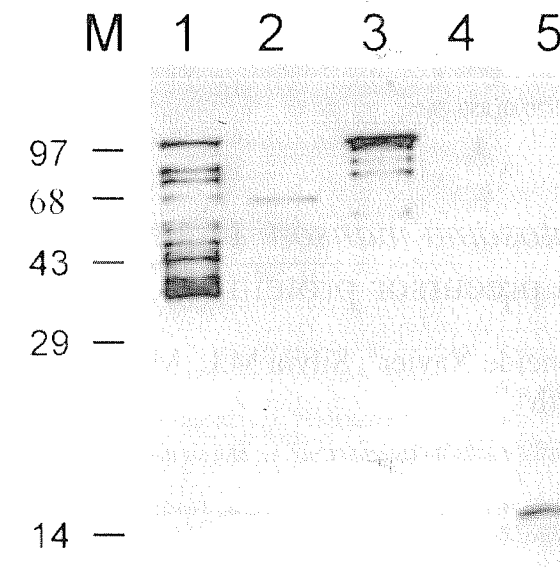


Fig. 1. Western blot probed with A70 R α M, specific to Sm15. 1, GST-fusion protein 2TR7A; 2, GST-fusion protein A70H1-G2; 3, GST-fusion protein A70-20; 4, GST; 5, *Schistosoma mansoni* adult worm tegumental membranes. M, molecular mass markers (kDa).

defining the amino terminus of Sm15. Since it is known that Sm15 has approximately 136 residues [3] this antigen was tentatively mapped to amino acids 362–497 (Fig. 2). Due to the repeat structure of the precursor, the stretch encompass-

ing amino acids 692–788 shares some epitopes with the segment defined by amino acids 362–497 (Fig. 2), indicating that some epitopes of Sm15 are part of the region of repeats. To our knowledge only one other group of tegumental proteins, the A1.12/9 antigen family, expressed in most life cycle stages of *S. mansoni*, has been shown to be differentially expressed [6]. These findings are important as they raise the possibility that proteolytic processing of tegumental proteins may be involved in the mechanisms leading to protective immunity or immune evasion in schistosomiasis [7]. In order to fully elucidate the biosynthesis and proteolytic processing of the Sm15 precursor, attempts to raise specific antibodies against different parts of the precursor are being pursued, so that other products of the precursor can be identified and their immunological role in schistosomiasis evaluated.

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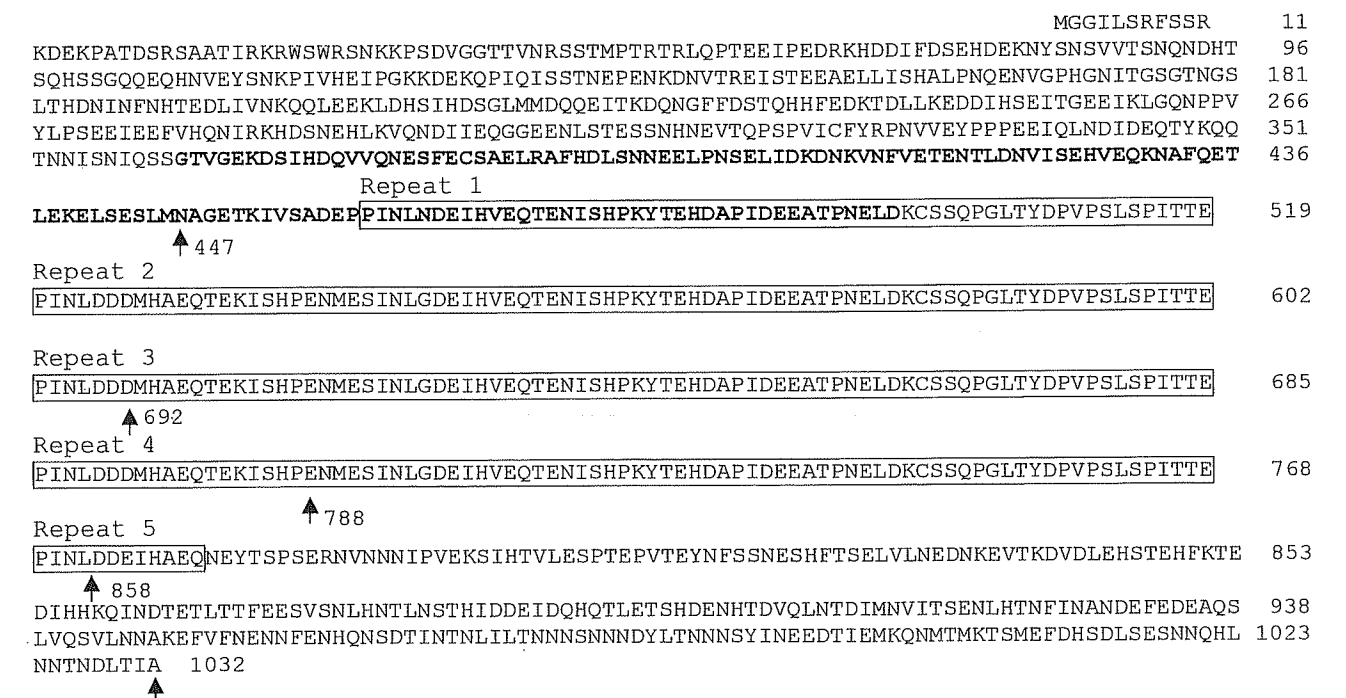


Fig. 2. Derived amino acid sequence of the Sm15 precursor (GeneBank™, accession number L07513, see Ref. 3) showing the putative mapping of Sm15 (in bold) and the region of repeats (boxed). The amino acids that define the position of the fusion proteins 2TR7A (amino acids 447–788), A70H1-G2 (amino acids 692–852) and A70-20 (amino acids 692–1032) are indicated by arrows.