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Molecular cloning and characterization of a 21 kDa protein secreted from *Trichinella pseudospiralis*

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Abstract

Recombinant protein was produced from the cDNA library of *Trichinella pseudospiralis*, which seemed to form part of the excretory–secretory (ES) products. The library was constructed from cDNA of muscle larvae at 1 month post-infection, and immunoscreened with antibody against *T. pseudospiralis* ES products. A clone, designated Tp21-3, contained a cDNA transcript of 657 bp in length with a single open reading frame, which encoded 172 amino acids (19617 Da in the estimated molecular mass). The predicted amino acid sequence of clone Tp21-3 had a similarity of 76% to that of clone ORF 17.20 (GenBank under accession number U88239) from *T. spiralis*. The recombinant fusion proteins encoded by clone Tp21-3 were produced in an *Escherichia coli* expression system and affinity purified. On Western blotting analysis, Tp21-3 recombinant proteins migrated at 40 kDa and reacted to antibody against *T. pseudospiralis* ES products and *T. pseudospiralis*-infected sera. Sera were developed against Tp 21-3 recombinant proteins, which reacted to a single band migrating at 21 kDa in crude worm extract and ES products from *T. pseudospiralis* on Western blotting analysis, and reacted with stichocytes of *T. pseudospiralis* on immunohistochemical staining.

Introduction

The genus *Trichinella* is a parasitic nematode in skeletal muscle cells of a wide variety of vertebrate hosts. Infection occurs by eating contaminated muscles which contain infective larvae. In the host stomach, infective larvae, which are released with the aid of host gastric juice, develop into adult worms in the host intestine in a couple of days. From 5 days post-infection (PI), the gravid female begins to produce a second generation of larvae, which penetrate the host tissue and migrate through the body of the host through blood and lymphatic vessels.

There are phenotypic differences between two species in the genus *Trichinella*, *T. spiralis* and *T. pseudospiralis*. The latter is smaller than the former (Bove *et al.*, 1979), and *T. spiralis* forms a typical cyst involving muscle cell transformation, whereas *T. pseudospiralis* does not form a cyst wall involving muscle cell transformation (Despommier, 1983; Xu *et al.*, 1997). Cyst formation is complete at about 18 days PI. The larvae in the cyst can be the source of the next infection. One unique feature of a *Trichinella* infection is muscle cell transformation to the nurse cell in the cyst (Despommier *et al.*, 1990; Lee *et al.*, 1991). Muscle cell transformation is likely to be initiated by excretory–secretory (ES) products released from the larvae (Ko *et al.*, 1994). To understand the mechanisms of such a transformation, ES products of *Trichinella* have received a great deal of attention. The major antigen recognized by the host during infection is attributed to the TSL-1 antigen in larval ES products of *T. spiralis* (Appleton *et al.*, 1991; Denkers *et al.*, 1991). Furthermore, genes encoding 43 kDa (Su *et al.*, 1991; Vassilatis *et al.*, 1992) and 53 kDa (Zarlenga & Gamble, 1990) ES products of *T. spiralis* have been characterized.

Although the ES products of *T. pseudospiralis* are likely to be involved in immunosuppression (Alkarmi & Faubert, 1981; Stewart *et al.*, 1985, 1988), the antigenic

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composition of ES products of *T. pseudospiralis* has not been well defined. In ES products of *T. pseudospiralis*, genes encoding 43 kDa and 23 kDa immunodominant antigens only have been characterized (Vassilatis *et al.*, 1996; Chung & Ko, 1999).

The ES products are composed of a wide variety of proteins. Therefore, it is necessary to determine each role endowed to individual protein components. For this purpose each component of the ES products should be mass isolated with a high degree of purity. In this paper we investigate the production of approximately 21 kDa recombinant fusion protein of *T. pseudospiralis*.

Materials and methods

Parasites, crude extracts and ES products

Larvae of *T. pseudospiralis* (ISS13) at the muscle stage were isolated from mice at 2 months PI using pepsin-HCl digestion. Crude saline extracts of larvae and ES products were prepared by conventional methods (Wu *et al.*, 1998; Wakelin *et al.*, 1994).

Preparation of antisera

Infected sera were obtained from BALB/c mice infected with 300 larvae of *T. pseudospiralis* for 2 months. Polyclonal antibodies against larval ES products were collected from outbred Wistar rats injected intradermally with approximately 200 μg of ES products and complete Freund's adjuvant followed by four booster injections of 100 μg of protein mixed with incomplete Freund's adjuvant at 2-week intervals. Antiserum against the recombinant protein was produced in BALB/c mice adopting a similar method with an injection dose of 100 μg fusion protein.

Preparation of T. pseudospiralis *cDNA libraries*

Muscle larvae of *T. pseudospiralis* from mice at 1 month PI were isolated and washed extensively with diethylpyrocarbonate-phosphate buffered saline (PBS). Poly (A)-rich RNA was isolated and purified using a Quickprep micro mRNA purification kit (Amersham Pharmacia Biotech, Tokyo Japan), and cDNA was prepared using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech) as described by the manufacturer. After addition of *Eco*R I adaptor, the cDNA were ligated into a λZAP II vector (Stratagene, La Jolla, California USA) with dephosphorylated *EcoR* I overhangs, and packaged in Gigapack Gold III packaging extract (Stratagene).

Immunoscreening of T. pseudospiralis *cDNA library and cloning of cDNA clone*

The *T. pseudospiralis* cDNA library was immunoscreened with a 1:100 dilution of polyclonal antibodies against *T. pseudospiralis* ES products according to the conventional methods (Sambrook *et al.*, 1989). Some of the positive cDNA clones were converted to a plasmid by *in vivo* excision, and each plasmid was propagated in an *E. coli* strain SOLR. The *Eco*R I (New England BioLabs, Beverly, Massachusetts, USA) restriction fragments of the purified plasmid from the clones were subcloned into the pET-32 expression vector (Novagen Inc., Madison, Wisconsin, USA) as described by Yamasaki *et al.* (1998).

Expression and purification of recombinant protein

The recombinant plasmid was transformed into an *E. coli* BL21(DE3)pLysS strain, and the expression of a polyhistidine-containing recombinant proteins was induced by adding β-D-thiogalactopyranaside (IPTG) at a final concentration of 1 mM at 37°C for 2–3 h. The induced cells were harvested and disrupted by sonication in 20 mM Tris-HCl buffer (pH 8.0). Fusion proteins expressed as inclusion bodies were solubilized completely with 6 M guanidine hydrochloride in 20 mM Tris-HCl buffer (pH 8.0), and then subjected to a His Trap kit for affinity purification of histidine-tagged proteins according to the manufacturer's instructions. Guanidine hydrochloride was removed from the samples with a PD-10 column (Amersham Pharmacia Biotech). Recombinant proteins thus obtained were analysed with 11% sodium dodecyl sulphate (SDS)-polyacryamide gel electrophoresis (PAGE) to assess their purity.

DNA sequencing

The recombinant plasmid isolated from the *E. coli* SOLR strain was sequenced using an automatic sequencer. The DNA sequences were assembled and analysed using the DNASIS software (Hitachi software engineering, Tokyo, Japan). The BLAST network service was used to search the DNA and protein database at the National Center for Biotechnology Information (Bethesda, Maryland, USA).

Western blotting analysis

Protein samples were electrophoresed on 11% SDS-PAGE then either stained with Coomassie brilliant blue G-250 or electrophoretically transferred to nitrocellulose sheets as described by Towbin *et al.* (1979). After transfer, nitrocellulose filters were blocked overnight at 4°C in 5% non-fat dried milk in PBS, and then incubated with the primary antibody preabsorbed with *E. coli* lysate, diluted 1:100. The primary antibody included either *T. pseudospiralis*-infected sera, rat polyclonal antibody against *T. pseudospiralis* ES products, or antiserum against the recombinant protein (see 'Preparation of antisera'). Antibodies conjugated with alkaline phosphatase were used as the second antibodies and the alkaline phosphatase developed in 5-bromo-4-chloro (3 indolyl) (10-toluidine) salt and nitroblue tetrazolium.

Immunohistolocalization

Skeletal muscle tissues from mice 22 days PI with *T. pseudospiralis* were immediately frozen and cryosectioned. Sections of 4 μm in thickness were incubated in a humid chamber with the anti recombinant protein antibody (1:100 dilution) for 1 h, washed, and further processed using the HistoStain SP kit (ZYMED Laboratories, Inc. South San Francisco, California, USA) according to the manufacturer's instructions.

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Tp21-3       1:MHCQYILSLLLLSLNVVFFAAGDSLDSVDDKSRRCTDEQTEVCAKTECKAEDAAMTELLL
ORF17.2Ø     1:....F....VFF...T...T......AIN..LK...EK...I..Q...........D...
               **** ****   *** *** ******   **  ***  *** ** *********** ***

Tp21-3      61:EGESDITEHPDFVYYTRCMQRCCAKLNGAKVAPLKEEEKRRGPTKLPFQSIFDVADQQTV
ORF17.2Ø    61:......FD.S..TS.AT..H....R....A.P....G......S........E....K..
               ******  * **  *  ** **** **** * **** ****** ******** **** **

Tp21-3     121:ERCDATMCKSQRMKYESLVARTTSYKKLRASQELRDYKECIESCDAKLNGRQ  172
ORF17.2Ø   121:....E.....H.Q...N.....S......S....K.......R.......--  17Ø
               **** ***** * *** ***** ****** **** ******* *******
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Fig. 1. Alignment of the deduced amino acid sequence of the Tp21-3 open reading frame with ORF17.20 (GenBank under accession number U88239). The asterisks indicate amino acids that are identical to those of Tp21-3 and ORF17.20. The numbers along the margin and at the end of the last line designate the positions of amino acid residues.

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been submitted to the GenBank™, EMBL and DDBJ databases and has the accession number AF269089

Results

Molecular characterization of clone Tp21-3

Primary immunoscreening of the cDNA expression library (200000 plaques) with infected mouse sera resulted in 80 positive clones. Plaques were picked up and rescreened with polyclonal antibodies against the ES products, and 30 positive clones were converted to a plasmid by *in vivo* excision. Plasmids were purified and digested by the restriction endonuclease *Eco*R I. Differences of length and restriction patterns using the restriction enzyme of inserted fragments showed that 20 of 30 positive clones overlapped each other (data not shown). One clone of the 20 clones, designated Tp21-3, was sequenced and its amino acid sequence deduced. The clone Tp21-3 consisted of 657 bp including a 3′ and 5′-untranslated region. The sequence of the predicted open reading frame encoded a protein of 172 amino acid residues with a molecular mass of 19617 Da. The initiation codon of methionine was expected to be positioned at 8–10 of the cDNA. A database search revealed that eight protein sequences were similar to the predicted protein. The best score identified was a clone from *T. spiralis*, which was ORF 17.20 reported by Polvere *et al.* (GenBank under accession number U88239). The similarities of the amino acid and DNA sequence of the open reading frame between the Tp21-3 and ORF 17.20 were 76% and 87%, respectively. The alignment of the deduced amino acid sequence of Tp21-3 and ORF 17.20 is shown in fig. 1.

Expression of clone T p21-3

Although the protein Tp21-3 is 19617 Da, it migrated at 40 kDa on SDS-PAGE due to the possession of 20 kDa plasmid vector proteins. The Tp21-3 recombinant protein was much more highly expressed in inclusion bodies than in the supernatant of induced cells. Protein synthesis was inducted more efficiently in the sample with 1 mM IPTG treatment than in the one without treatment. The recombinant protein could be purified at a single band level using a His Trap kit and eluted with 500 mM imidazole.

Western blotting analysis of recombinant protein

The Tp21-3 recombinant protein migrated at 40 kDa, which were positively immunostained with anti-*T. pseudospiralis* ES sera (lane 2 in fig. 2) and *T. pseudospiralis*

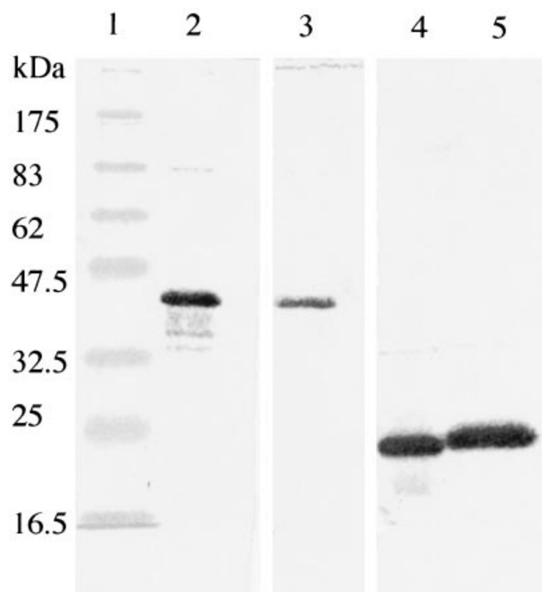

Fig. 2. Western blotting analysis of Tp21-3 recombinant protein with antisera against *Trichinella pseudospiralis* ES products (lane 2) and the *T. pseudospiralis* infected sera (lane 3). Western blotting analysis of crude worm extract (lane 4), ES products (lane 5) from *T. pseudospiralis* with antibody against Tp21-3 recombinant protein. Lane 1: molecular weight standard, size in kDa is shown on the left side.

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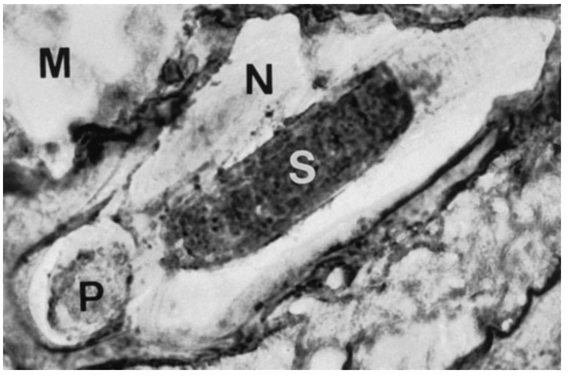

Fig. 3. *Trichinella pseudospiralis* on 22 day PI in the host muscle immunostained with anti-Tp21-3 antibody. Positive immunoproducts are seen on stichocyte (S). N, nurse cell; M, host muscle cell; P, parasite.

infected sera (lane 3 in fig. 2). Anti-Tp21-3 sera detected a strong band migrating at 21 kDa in crude worm extract (lane 4 in fig. 2) and in ES products (lane 5 in fig. 2) from *T. pseudospiralis*.

Histolocalization of the recombinant protein in infected skeletal muscle

Intense staining with the anti-Tp21-3 serum was found within the stichocyte of muscle larvae of *T. pseudospiralis* at 22 days PI (fig. 3).

Discussion

In this study we have established a cDNA clone named Tp21-3 that encodes the 19617 Da protein of *T. pseudospiralis*. The recombinant protein encoded by the clone Tp21-3 was successfully produced in the *E. coli* expression system, and some profile of the fusion protein was revealed.

The Tp21-3 protein is part of the ES products of *T. pseudospiralis*, as can be concluded from the following evidence. Firstly, the clone was selected by immunoscreening with anti-ES sera. Secondly, on Western blotting, the recombinant protein was positively immunostained with the same anti-ES sera. Thirdly, the antibody against the Tp21-3 protein recognized the 21 kDa band of ES products on Western blotting and positively immunostained stichocytes of *T. pseudospiralis* muscle larvae.

Previously Wu *et al.* (1998), using two-dimensional (2-D) electrophoresis, showed that there were a number of peptide spots in the ES products of *T. pseudospiralis*, migrating at 20–90 kDa. But ES products of *T. pseudospiralis* recognized by infected sera migrated mainly at 35 and 45 kDa and very few at 20 kDa (Wu *et al.*, 1999). This may be due to the *Trichinella* infection inducing only weak antibody responses to the Tp21-3 antigen in mice (see lane 3 of fig. 2), but that immunization results in strong antibody responses against Tp21-3 (see lane 2 of fig. 2).

The ES products are known to contain some functional proteins such as heat shock proteins, endonucleases, serine proteases and DNA-binding proteins (Ko & Fan, 1996; Mak & Ko, 1999; Moczon & Wranicz, 1999; Ko & Mak, 1999). These functional proteins have been produced by genetic engineering methods due to their potential for pharmacological use.

The function of the Tp21-3 protein is interesting but so far unknown. Database searches have not identified any related protein with a significant homology except for the clone of ORF17.20 whose function is also undetermined. Biochemical analysis also failed to show a proteinase or proteinase inhibition activity of the recombinant protein Tp21-3 (data not shown), whereas the ES products of *T. spiralis* possess proteinase and proteinase inhibition activity (Todorova *et al.*, 1995; Nagano *et al.*, 2001).

The two species, *T. spiralis* and *T. pseudospiralis* share ES products with considerable similarity, in terms of cDNA sequence, molecular weight and antigenicity (Kehayov *et al.*, 1991; Zhang *et al.*, 1993; Vassilatis *et al.*, 1996; Wu *et al.*, 1998, 1999). The protein encoded by clones Tp21-3 of *T. pseudospiralis* and ORF17.20 of *T. spiralis* seem to be one such example, because the homology of cDNA between the Tp21-3 and ORF 17.20 is 87%. Shared protein in ES products are likely to play a fundamental role common to the two species of *Trichinella* and therefore crucial for worm establishment in the host.

Reportedly, the 43 kDa protein is another example of the shared protein. The 43 kDa protein was first reported to be unique to *T. spiralis* (Almond *et al.*, 1986; Jasmer, 1990), but Wu *et al.* (1998) showed that the 43 kDa protein is shared by the two species of *Trichinella*, and both species express the cDNA encoding of the 43 kDa protein. Furthermore, Vassilatis *et al.* (1996) showed that the genome of *T. pseudospiralis* encodes sequences similar to the 43 kDa protein genome of *T. spiralis*. The 53 kDa protein reported by Zarlenga & Gamble (1990) is also not unique to *T. spiralis*, because cDNA encoding 53 kDa protein is expressed by both species (in our unpublished data).

Trichinella spiralis and *T. pseudospiralis* infections cause similar but different pathological changes (Matsuo *et al.*, 2000). Both species cause muscle cell degeneration, which is restricted around the worm in the case of *T. spiralis* infection but spreads over the entire length of muscle cell in the case of *T. pseudospiralis* infection. Both species cause satellite cell proliferation. Subsequent cell fusion is seen in *T. spiralis* infection but not in *T. pseudospiralis* infection. This difference affords a good experimental model to study molecular mechanisms responsible for muscle cell differentiation, and in such experiments ES products of *Trichinella*, including Tp21-3 fusion protein, can be used as a prohibitor of normal muscle cell repair.

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