AN ANTIGENIC PEPTIDE OF MYOSIN HEAVY CHAIN-LIKE PROTEIN FROM *TRICHINELLA SPIRALIS*

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Abstract: We produced antigenic peptide of *Trichinella spiralis* newborn larvae (NBL) which seemed to be a part (approximately one fourth) of myosin heavy chain, and some basic profiles were revealed. The cDNA library was constructed from NBL and immunoscreened with an antibody against the parasite. A clone, designated NBL21, was selected. It contained a cDNA transcript of 1656 bp in length, which encoded 552-amino acids (64868 Da in the estimated molecular weight). The fusion protein encoded by the clone NBL21 was produced in an *Escherichia coli* expression system and affinity purified. NBL21 fusion proteins migrated at 64 kDa and reacted to *T. spiralis*-infected mouse sera and the antibody against NBL crude antigen. Antisera were developed against NBL21 fusion proteins, which reacted to a single band migrating at 200 kDa on Western blotting analysis of crude extracts from muscle larvae, and reacted to hypodermal muscles of *T. spiralis* on immunohistochemical staining. The antigen was recognized by the mouse serum obtained from the early phase of infection, but the antigenicity was devoid of species specificity.

Key words: Myosin heavy chain, *Trichinella spiralis*, Antigen, Fusion protein

INTRODUCTION

Trichinella spiralis is a parasitic nematode that infects the skeletal muscle cells of mammals. Many questions remain to be answered about this parasite. Examples include how they evade host immune attack, and how they can establish parasitism in the host muscle cell. Moreover, the vaccine against this parasite is still far from routine usage.

For progress towards these goals, fusion proteins of *Trichinella* have been produced with some success. Vaysier *et al.* (1999) produced a protein from *T. britovi*, named as Tb Hsp70, which is a kind of heat shock protein with a molecular weight of 70 kDa. Sun *et al.* (1994) produced a protein Ts39 FP which is a potentially valuable antigen for vaccine development. Lindh *et al.* (1998) produced fusion protein which has properties of a myogenic repressor, and Nagano *et al.* (2001a) produced fusion protein with serine protease inhibitor activity.

In this study we produced a part of myosin heavy chain-like protein, and revealed some basic profiles including amino acid sequence and antigenicity. This is the first report on antigenic peptide of *Trichinella* myosin heavy chain which may afford better understanding of this conservative protein in *Trichinella* related research.

MATERIALS AND METHODS

Parasites

T. spiralis (ISS 413) and *Trichinella pseudospiralis* (ISS 13) were maintained in mice through oral infection. Muscle larvae were obtained by a conventional digestion method (Despommier, 1975). Newborn larvae (NBL) of *T. spiralis* were obtained through incubation of five day old adults in minimum essential medium (MEM) + 10% fetal calf serum (FCS) + antibiotics (100 μ l of streptomycin and 100 unit of penicillin per 100 ml of MEM). The NBL were frozen and stored until use.

Crude extracts and ES products

Larvae of *Trichinella* at the muscle stage were isolated from mice at 2 months post infection using digestion method. Crude saline extracts of larvae and ES products were prepared by conventional methods (Wakelin *et al.*, 1994; Wu *et al.*, 1998).

Preparation of antisera

Polyclonal antibodies against crude extracts of the NBL were collected from BALB/c mice injected subcutaneously with 30 μ g of the crude extracts and Freund's complete adjuvant followed by two booster injections of 15 μ g of the crude extracts mixed with Freund's incomplete adju-

vant at 2-week intervals.

Infected sera of the parasites and polyclonal antibodies against ES products were prepared by conventional methods (Nagano *et al.*, 2001a, b).

cDNA library construction

Construction of a cDNA library from *T. spiralis* NBL was performed according to our previous method (Nagano *et al.*, 1999). In brief, frozen NBL were homogenized in an extraction buffer (Quickprep micro mRNA purification kit, Amersham Pharmacia Biotech, Tokyo Japan), and mRNA was isolated by affinity chromatography using oligo (dT) cellulose.

Synthesis of cDNA from the resulting mRNA was performed using a Timesaver cDNA synthesis kit (Amersham Pharmacia Biotech) as described by the manufacturer's instructions. Resulting cDNAs were ligated into the λ ZAP II vector (Stratagene, La Jolla, California USA). The ligates were packaged in Gigapack Gold III packaging extract (Stratagene).

Immunoscreening

E. coli XL1-Blue MRF' strain infected with the λ ZAP II vector was cultured on the NZY agar plates for 4 hr at 42 °C. And nitrocellulose membrane infiltrated with isopropyl- β -D-thiogalactopyranoside (IPTG) was overlaid for 4 hr at 37 °C. After blocking with 5% skim milk in phosphate buffered saline (PBS) overnight at 4 °C, the membrane was reacted with polyclonal antibodies (1/100 diluted) against *T. spiralis* NBL. Goat anti-mouse IgG alkaline phosphatase conjugate was then used as the second antibody, and the membrane was incubated in 5-bromo-4-chloro-3-indolyl-p-toluidine salt (BCIP) and nitroblue tetrazolium (NBT) for development. The positive 66 clones were picked up and re-screened three times.

DNA sequence and homology search

The clone NBL21 was subjected to sequencing in which the Thermo Sequenase Cycle Sequencing Kit (Amersham) with bi-directional sequencing protocol and IRD-800 and IRD-700 labeled primers were used. The products were sequenced using an automatic sequencer (Model LIC-4200 L, Aloka, Tokyo Japan). The DNA sequences were assembled and analysed by using DNASIS software (Hitachi Software Engineering, Tokyo Japan). The NCBI BLAST network service at the National Center for Biotechnology Information was used to search for the sequence homology.

Production of fusion protein

The NBL21 clone was subcloned into the expression vector (pTrc His, Invitrogen, California USA) and trans-

ected to *E. coli* (DH5 α , Toyobo CO. LTD., Tokyo Japan) according to the manufacture's instructions. Transformants were grown in bacterial culture medium at 37 °C. To facilitate protein production, IPTG was added to give a final concentration of 1 mM, and cells were incubated for 3 hr at 37 °C. The induced cells were fractured by ultrasound in 20 mM Tris HCl buffer (pH 8.0). The resultants were precipitated and the pellet was lysed by lysis buffer (20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride). The fusion protein was affinity purified using a HisTrap kit (Amersham Pharmacia Biotech) as described by the manufacturer.

Production of antibodies against the fusion protein

To produce antibodies against the fusion protein NBL 21, BALB/c mice were injected subcutaneously with 30 μ g of the fusion protein and Freund's complete adjuvant. In order to boost the antibodies, after two weeks, the mice were injected with 15 μ g of the protein in Freund's incomplete adjuvant, and after another one week, 10 μ g of the protein was injected into the abdominal cavity. The mice were bled one week after the final booster.

Immunocytochemistry

Infected muscles of 42-day post infection were fixed with 4% paraformaldehyde and embedded in OCT compound (Miles Inc., Elkhart, Indiana USA) and frozen. Cryostat sections (4 μ m in thickness) were transferred to albumin coated microscope slides. The sections were immunostained using Histostain SP kit (Zymed Laboratories Inc., San Francisco, California USA) as described by the manufacturer's instructions. Briefly, the sections were treated with the first antibodies ($\times 100$ diluted antibody against the NBL21 fusion protein or control normal mouse serum) for 30 min at room temperature. After washing, the sections were treated with the biotinylated second antibody (Zymed Laboratories Inc.) for 30 min at room temperature, washed three times, treated with avidin coupled with peroxidase (Zymed Laboratories Inc.) for 10 min at room temperature, and reacted with diaminobenzidine solution (Zymed Laboratories Inc.) for peroxidase staining.

Antigenicity analysis of the fusion protein NBL21

The fusion protein NBL21 was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted to nitrocellulose membranes. Membranes were blocked with skim milk and reacted with the first antibodies ($\times 100$ diluted) including anti NBL antibodies, infected sera, antibodies against excretory and secretory (ES) products of muscle larvae, and control sera for 60 minutes at 37 °C. After washing, the membranes were treated

with alkaline phosphatase-conjugated anti-mouse IgG for 30 min at 37°C. The alkaline phosphatase was developed by using BCIP and NBT.

Specificity analysis of antibody against the fusion protein NBL21

Reactivity of anti-NBL21 fusion protein antibody was checked against 4 kinds of antigens including crude antigens and ES products from the two species (*T. spiralis* and *T. pseudospiralis*). The antigens were subjected to SDS-PAGE and reacted to the anti NBL21 antibodies ($\times 100$ diluted) for 60 min at 37°C. The nitrocellulose membranes were treated in the same way as described above.

Specificity of fusion protein NBL21

Antigen specificity of the fusion protein was assessed in two ways. The panel of crude antigens of parasites (*Dirofilaria immitis*, *Ascaris lumbricoides*, *Toxocara canis*, *Gnathostoma nipponicum*, *Sparganum mansoni*, *Clonorchis sinensis*, *Anisakis simplex*, *Strongyloides stercoralis*, *Paragonimus westermani*, *Paragonimus miyazakii*, *Fasciola hepatica* and *Cysticercus cellulosae*) was subjected to reaction against anti fusion protein NBL21 antibody by multiple dot ELISA according to the established method. The other method included ELISA where fusion protein NBL21 was reacted against a panel of human patient sera (trichinosis, paragonimiasis, dirofilariasis, dihyalobothriasis, anisakiasis, cysticercosis cellulose hominis, trichuriasis, gnathostomiasis and fasciolosis) according to the established method.

Kinetics of antibody response against the fusion protein NBL21

Three BALB/c mice were each inoculated orally with 300 infective larvae. Sera from infected mice were collected before inoculation for a control and at 1, 2, 3, 4, 6, 8 and 10 weeks after inoculation. Antibody titer against fusion protein NBL21 was assessed by means of ELISA according to the established method.

Nucleotide sequence accession number

The nucleotide sequence reported in this article has been submitted to the GenBank database and has the accession number AY033438.

RESULTS

DNA and amino acid sequences

The cDNA library from NBL gave 66 positive clones, which comprised of 3 groups of clones. One clone, designated NBL21, from the major group was further subcloned. The clone NBL21 consisted of 1656 bp nucleotide and en-

coded 552 amino acid residues (Fig. 1). A homology study from NCBI BLAST search revealed significant homology with myosin heavy chain of some nematodes including *Caenorhabditis elegans* (GenBank under accession number X08067), *Onchocerca volvulus* (GenBank under accession number M74066) and *Brugia malayi* (GenBank under accession number L01625).

Myosin heavy chain of *C. elegans* had 65% homology in the DNA sequence and 66% in the deduced amino acid sequence. Myosin heavy chain of *O. volvulus* had 64% homology in the DNA sequence and 63% in the deduced amino acid sequence. Myosin heavy chain of *B. malayi* had 62% homology in the DNA sequence and 64% in the deduced amino acid sequence. In some areas such as amino acid sequence 260-293 (box in Fig. 1), more significant homology was found; 75-77% homology in the DNA sequence and 88-94% in the deduced amino acid sequence.

Western blotting

The fusion protein NBL21, produced by means of an *E. coli* expression system, was migrated at 68 kDa on SDS-PAGE and positively immunostained with anti *T. spiralis* NBL antibody (arrow in lane 1 of Fig. 2), *T. spiralis* infected sera (arrow in lane 2 of Fig. 2), *T. pseudospiralis* infected sera (arrow in lane 3 of Fig. 2), but not with anti ES product of *T. spiralis* muscle larvae (lane 4 in Fig. 2), anti ES product of *T. pseudospiralis* muscle larvae (lane 5 in Fig. 2) nor with control sera (figure not shown).

An SDS-PAGE analysis revealed that crude antigens and ES product of muscle larvae were composed of many bands with different molecular weights. Out of many bands, only one band of 200 kDa (corresponding to the expected size) of crude antigens of *T. spiralis* (lane 2 in Fig. 3) and *T. pseudospiralis* (lane 4 in Fig. 3) were positive by immunostaining with the antibody against NBL21 fusion protein. No bands of the ES product of the two species were positive by the immunostaining (lane 3, 5 in Fig. 3).

Immunolocalization

As shown in Fig. 4 A, strong positive immunostaining was observed in the internal structures of the muscle larvae. The staining consisted of a spotty pattern and lining beneath the cuticle surface, and internal organs were negative by the staining. The control sera failed to stain any structures (Fig. 4 B).

Antigen specificity

The multiple dot ELISA showed reactivity of the antibody against the fusion protein NBL21 against crude extracts of *D. immitis*, *A. lumbricoides*, *T. canis*, *G. nipponicum*, *S. mansoni*, *C. sinensis* but not against *A. simplex*, *S.*

NBL21 1:LNRLXPQYQTQLLEEAKRTADEEKNRHNLAQKLNMEHENQSRLREEEAESKTRHISKLNAEIQQ
C.ele 1314:H..KSTL.S..D.TR.NY...SR..QA...TA...L...TI...H.D....ADLT.Q.....
O.vol 1308:MT..KA.LTS.....R.SL...ARD.N....M..YQ..IEQVL.SM...I.GIS.LMKQL.RA.....
B.mal 664:MT..KA.LTS.....R.SL...ARD.N....S..YQ..IEQI..SM...I.A.N.LMKQL.RA.....
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NBL21 71:WKAFESEGLARVDEIEEAKRKLTQKVQMGEAFEANGKIASLEKIRHKLLGEIDDAQVDVERANNYYAA
C.ele 1384:...R.D...NKLE...A.KA.QL...LDTN.GLFA...Q.V.F.MQDL...S...K.AAQV
O.vol 1378:QTR.....LKG..L.S.KROMH.MN.L.TLD...S.S...TKSR.VSDL...M.....S.S
B.mal 734:QTR.....LKS..L.S.KROM..IN.L..LD...S.S...TKSR.VSDL...M.....S.S
* * **** * * * * ** * * * **** * *

NBL21 141:QLEKKQKGFDKIVDEWKKKCDDLSSSELDAQRENRLSTECFLKNSQDELIEQIEAVRRENKNLVQEIK
C.ele 1454:FY..HRRQ.ES.IA....T.....A..D..Q...DL..A.TAN...A.YLDST....S.A..V.
O.vol 1448:.....VI.....T..IAT.V.NA...A.NV...L...SE...VL.T..GL....E.A..R.
B.mal 804:L.....VI...R.T..ILA.V.NA...A.NV...L...SER..VL.T..GL....E.A..
** * **** * * ** * ** * * ** * **** * *

NBL21 211:DITDQLGEGGRSVHELQKVVRRLEREKEELQQALDEAESALEAESKVMRAQVEVSQIRQIEKRIREKE
C.ele 1524:L.....A...I..K..V...K.....A..L...I.....S.....Q...
O.vol 1518:L.....F.M..II...V..D..H...QPQD.....L.....A.....Q...
B.mal 874:L.....F.M..II...V..D..H...A.....L.....A.....Q...
* ***** * * * ** * ** * ***** ** *** ***** ***** *

NBL21 281:EFEENTRNKHQRALDSMQATLESEAKGRAEALRLKKKLESDINELEIALDHANKANADAQKNIKMYGDQV
C.ele 1594:.....R.....E.....A.T.QKE...I.....D.....R.Y.....T..K.MET.
O.vol 1588:.....IE...S..N.TRSK.DLM.....Q...Q...V.T...M
B.mal 944:.....IE...S..N.TRSK.DLM.....F.....Q...EV...V.R...I
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NBL21 351:KELQMHIEDERQREEIREQFHASEKRCAMLQSEKEYMTASEQAERARRQAEELYELREQVNLSSTN
C.ele 1664:Q...FQ..E...KD.....L...N..I...D.LAQA.A.....N...CI....N.D.NAHV
O.vol 1658:R...QQV.T...NGRNS..YLN..KATL.....MSV.NG.....K..DYDAN.AHT.C...AQ
B.mal 1014:R...QQV.I...N.....YLN..KATL.....TSV.MD...T.K.S.RDAN.AHV.C...AQ
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NBL21 421:ASLSAIKRKLEGELQALHAELDCTLNELKKVDEQCKKAMTDARLAELRQEQEHSMHVERMRKGLEQQV
C.ele 1734:SA.TGQR.....L.A...EEIA...NAV..GQ..SA.....I..I.....L..I
O.vol 1728:E..CGSR...DT..L.IQ.D.E...Y.ASE.R..A.SS.....Q..K..N.LQND.I..A..S.L
B.mal 1084:E...SV.K...T..L.IQ.D.E...Y.ASE.RY.A.SS.....Q.....N.LQND.I..A..S.L
* ** * * * * ** * * * ***** ** * * * *

NBL21 491:KEMQVRLDEAEQAALKGGKKIQKLEQRIRELEQELDLEQRRHQETDKNMQRKQDRRIKETDS
C.ele 1804:...I...D..N.....AQ..A..AI.....G.....D.E.W..AE..V..VEF
O.vol 1798:...A.....VL.....N.D.A..S.....S..G...Y...N.SLT.HE...R.LQF
B.mal 1154:...A.....A.....V.A..S.....S..G.R..Y...N.SLT.HE...R.LQF
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Figure 1 Comparison of a deduced amino acid sequence of the clone NBL21 and its comparable genes from other nematodes including *C. elegans* (C. ele; amino acid residues 1314-1865 in the total 1969 residues), *O. volvulus* (O. vol; amino acid residues 1308-1859 in the total 1957 residues), *B. malayi* (B. mal; amino acid residues 664-1215 in the total 1313 residues). The asterisks indicate nucleotides, which are identical to those of C. ele, O. vol, B. mal and NBL21. A box shows high homology region mentioned in the text.

Figure 2 Western blots analysis of the fusion protein NBL21. *E. coli* expressed NBL21 fusion protein was reacted with anti NBL21 antibody (lane 1), infected sera of *T. spiralis* (lane 2), infected sera of *T. pseudospiralis* (lane 3), anti ES product of *T. spiralis* antibody (lane 4) and anti ES product of *T. pseudospiralis* antibody (lane 5). The left side of each lane is molecular marker and the right side is the test sample. The numbers of the left side is molecular weight. The arrow shows positive band.

Figure 3 Western blot analysis of reactivity of anti NBL21. The mouse antibody produced against the fusion protein NBL 21 was reacted with NBL crude antigen (lane 1), *T. spiralis* crude antigen (lane 2), *T. pseudospiralis* crude antigen (lane 4), ES products of *T. spiralis* (lane 3) and ES products of *T. pseudospiralis* (lane 5). The numbers are molecular weights of marker (m).

Figure 4 Immunocytochemical staining of muscle larvae of *T. spiralis* with antibody against NBL 21 fusion protein (A) and control serum (B). The arrow shows the positive staining.

Figure 5 Dot - ELISA analysis of reactivity of anti NBL21 fusion protein antibody (S) and normal mouse sera (C) against a panel of crude antigens. 1. *P. westermani*, 2. *P. miyazakii*, 3. *F. hepatica*, 4. *C. sinensis*, 5. *S. mansoni*, 6. *C. cellulosae*, 7. *D. immitis*, 8. *T. canis*, 9. *A. lumbricoides*, 10. *A. simplex*, 11. *G. nipponicum*, 12. *S. stercoralis*. Crude antigens from *C. cellulosae*, *A. simplex* and *S. stercoralis* gave negative results which may suggest major difference in antigenic epitope of myosin heavy chain from other parasites.

stercoralis, *P. westermani*, *P. miyazakii*, *F. hepatica* and *C. cellulosa*. *D. immitis* and *T. canis* especially exhibited strong reactivity ("S" in Fig. 5). Normal mouse sera ("C" in Fig. 5) gave negative or weak positive result.

The fusion protein reacted positive for ELISA against sera from all patients examined including nematode, cestode and trematode infection (figures not shown).

Antibody response kinetics

Antibodies against the fusion protein were detected from 4 weeks after the infection and reached a maximum at 8 weeks, and the ELISA titer remained high throughout the 10-week-long experiment (figures not shown).

DISCUSSION

Recent progress in cell biology has enabled us to produce a large amount of proteins with high purity. Taking advantage of this methodology, proteins of a parasite origin have been mass-produced for a variety of usages including immunodiagnostic antigen (Chung and Ko, 1999) and vaccine (Sun *et al.*, 1994; Prema *et al.*, 1994). Genes encoding specific antigens of ES products (43, 39 and 53 kDa) have been characterized (Sugane and Matsuura, 1990; Zarlenga and Gamble, 1990; Su *et al.*, 1991; Vassilatis *et al.*, 1992).

In this study we obtained the clone NBL21 from *T. spiralis* NBL. This clone is likely a part of the myosin heavy chain from the sequence homology and in situ localization of this protein within the parasite. This study also revealed some basic profiles of the myosin heavy chain including the DNA and amino acid sequence which is discussed with comparable genes of other nematodes such as *C. elegans*, *B. malayi* and *O. volvulus*. Antigenic specificity and possible immunodiagnostic usages were also assessed.

Myosin is a fundamental protein shared by all kinds of cells. The DNA sequence of such a protein should be highly conservative. This notion is supported by the present study which showed high homology among other nematodes. In particular, some areas of the nucleotides are significantly conserved. Such area may be responsible for its common function as myosin.

The peptide NBL21 seems to carry immunodominant antigen which is recognized by hosts during the experimental infection. A similar result was reported by Li *et al.* (1995) who demonstrated that myosin heavy chain carries immunodominant filarial antigen. The clone NBL21 had 48% homology with the myosin heavy chain of hosts (humans and mouse), unshared sequence may explain the reasons why the fusion protein NBL21 is highly antigenic against the hosts.

We have no ready explanation as to how this internal

antigen is exposed to the host immune system. Interestingly, paramyosin, which is also an internal constituent of parasites, carries immunodominant antigen which may be used as a protective antigen against schistosome infection (Xu *et al.*, 1989). Our preliminary experiment, however, showed that the fusion protein NBL21 does not provoke protective immunity against challenge infection.

Fusion proteins of parasites can be a candidate for immunodiagnostic antigen as suggested by many authors (Zarlenga and Gamble, 1990; Chandrashekar *et al.*, 1991; Zarlenga *et al.*, 1994). The mice infected with *Trichinella* seem to provoke antibodies at detectable levels against the fusion protein NBL21 from the early phase of the infection, therefore, the antigen of the fusion protein NBL21 seems to be Group I antigen (Denkers *et al.*, 1990) and rapid responding group antigen (Takahashi *et al.*, 1990). One drawback of this fusion protein is its lack of species specificity. The antibody against the fusion protein NBL21 reacted against sera from a variety of parasite crude antigens. Therefore, this fusion protein can be used for early diagnosis of some parasite infection.

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