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Short communication

Molecular characterisation of a cognate 70 kDa heat shock protein of the protozoan *Theileria parva*¹

Claudia Daubenberger^{a,*}, Volker Heussler^a, Elke Gobright^a, Peter Wijngaard^b,
Hans C. Clevers^b, Clive Wells^a, Naotoshi Tsuji^c, Anthony Musoke^a,
Declan McKeever^a

^a International Livestock Research Institute (ILRI), P.O. Box 30709, Nairobi, Kenya

^b Department of Immunology, University Hospital, 3584CX Utrecht, The Netherlands

^c 2nd Protozoology Laboratory, National Institute of Animal Health, 3-1-1 Kannondai, Tsukuba, Ibaraki 305, Japan

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Theileria parva, a tick-transmitted protozoan parasite, infects cattle in eastern, central and southern Africa, leading to an acute, usually fatal lymphoproliferative disorder known as East Coast fever [1]. It has been shown that the protective immune response against *T. parva* is mediated by

class I MHC-restricted CD8⁺ cytotoxic T lymphocytes (CTL) recognising schizont transformed lymphoblasts (TpM) [2]. Antigens expressed by TpM that constitute targets for CTL have yet to be identified. Therefore, a library was constructed from Poly(A)⁺ RNA derived from a TpM cell line, D409/N2 [3]. From this library, several colonies carrying parasite encoded genes were identified and are currently being characterised. Since heat shock proteins (hsp) of many infectious agents are known to constitute dominant antigens that may play an important role in the host-parasite relationship [4], we focused our attention on a newly cloned hsp gene, with the aim of elucidating its relevance to *T. parva* immunity.

Screening of the D409/N2 library using total genomic DNA obtained from piroplasms of *T.*

Abbreviations: CTL, cytotoxic T lymphocyte; GST, glutathione-S-transferase; hsp, heat shock protein; hs, heat shock; RT-PCR, reverse transcriptase polymerase chain reaction; TpM, *T. parva* schizont-infected cell line.

* Corresponding author. Present address: Swiss Tropical Institute, Postfach, CH 4002 Basel. Tel.: +41 61 2848111; fax.: +41 61 2718654.

¹ Note: Nucleotide sequence data reported in this paper have been submitted to the GenBank™ data base with the accession number U40190. This is ILRI publication number 96042.

	10	20	30	40	50	60	70
<i>T. parva</i>	MT--GPAIGI	DLGTTYSCVA	VYKDNVVEII	PNDQGNRTTP	SYVAFTDTER	LIGDAAKNQE	ARNPENTIFD
<i>Bos taurus</i>	*-SK***V**	*****G	*FQHGK****	A*****	*****	*****V	*M**T**V**
	80	90	100	110	120	130	140
<i>T. parva</i>	AKRLIGRKFD	DRTVQEDMKH	WPFKVTNGPN	GKPNIEVTFQ	GEKKTFFHAE	ISSMVLTKMK	EIAEAFGLKS
<i>Bos taurus</i>	*****R**	*AV**S****	***M*V*DAG	-R*KVQ*EYK	**T*S*YP**	V*****	*****Y***T
	150	160	170	180	190	200	210
<i>T. parva</i>	VKDVTITVPA	YFNDSSQRQAT	KDAGTIAGLN	VMRIINEPTA	AAIAYGLDKK	GGGEKNVLIF	DLGGGTDFVS
<i>Bos taurus</i>	*TNA*V****	*****	*****	*L*****	*****	V*A*R*****	*****
	220	230	240	250	260	270	280
<i>T. parva</i>	ILTIEDGIFE	VKATAGDTHL	GGEDFDNLLV	EHCVRDFMRL	NNGKNISSNK	RALRLRTHC	ERAKRVLSSS
<i>Bos taurus</i>	*****	**S*****	*****RM*	-NHFAIEFKR	KHK*D**E**	**V*****A*	*****T****
	290	300	310	320	330	340	350
<i>T. parva</i>	TQATIELDSL	YEGIDYNTTI	SRARFEELCN	EKFRSTLVPV	EKALESSGLD	KRSIHEVVLV	GGSTRIPKIQ
<i>Bos taurus</i>	***S**I***	*****FY*S*	T*****NA	DL**G**D**	*****RDAK**	*SQ**DI***	*****
	360	370	380	390	400	410	420
<i>T. parva</i>	TLIKNFFNGK	EPCRSINPDE	AVAYGAHVQA	AILSGNQSEK	IQELLLLDVA	PLSLGLETAG	GVMTVLIKRN
<i>Bos taurus</i>	K*LQD*****	*LNK*****	*****	*****DK**N	V*D*****T	*****I****	*****
	430	440	450	460	470	480	490
<i>T. parva</i>	TTIPTKKNQI	FTTNEDRQEG	VLIQVFKGER	AMTKDNNLLG	KFHLTGIAPA	PRGVPQIEVT	FDIDANGILN
<i>Bos taurus</i>	*****QT*T	***YS*N*P*	*****YE***	*****	**E*****P**	*****	*****
	500	510	520	530	540	550	560
<i>T. parva</i>	VTAMDKSTGK	SEHVTITNDK	GRLSQEEIDR	MVEEAKEYKE	EDEKRRKCEV	SKHKLENYCY	SMKNTLSEDQ
<i>Bos taurus</i>	*S*V*****	ENKI*****	****K*D*E*	**Q*****A	****Q*DK*S	**NS*KS*AF	N**A*VEDEK
	570	580	590	600	610	620	630
<i>T. parva</i>	VKQKLGADEV	DNALNTITEA	LKWVETNQLA	EHDEFEDKLL	HVEGVCNPLV	TKLYQSGGAP	GAGPDMGAG-
<i>Bos taurus</i>	LQG*INDEDK	QKI*DKCN*I	IN*LDK**T*	*KE***HQQ*	EL*K*****II	*****A*GM	PG*MPG*MPG
	640	650					
<i>T. parva</i>	-FPGGAPPPS	S--SSGPTVE	EVD*				
<i>Bos taurus</i>	G****GA***	GGA*****I*	****				

Fig. 1. Comparison of the deduced amino acid sequence of X88 cDNA clone and the sequence of a bovine 70 kDa heat shock cognate protein [8]. The gaps (shown as dash) have been introduced to maximise homology. The conserved sequences near the N-terminus and at the extreme C-terminal end are underlined.

Fig. 2. (A) Analysis of relative changes in X88 mRNA levels by competitive RT-PCR. Total RNA from TpM and hsTpM (TpM were kept for 2h at 42°C) was purified as described [16] and first strand cDNA synthesised using a Reverse Transcription System (Promega). Ten per cent of the cDNAs from TpM (I) and hsTpM (II) were co-amplified in the presence of serial dilutions of competitor DNA (in attomoles) containing identical primer binding sites as X88. Competitor DNA was constructed with the PCR MIMIC™ construction kit (Clontech). PCR-reactions were performed containing 5 mM each of primer A (5' TTC TAG AAT GAC AGG ACC AGC A 3') and B (5' TTA GTC CAC CTC CTC AAC AGT T 3') with 30 cycles of incubation for 2 min at 94°C, 2 min at 55°C and 2 min at 74°C. One tenth of the PCR products was resolved on a 1.5% (w/v) Ethidium bromide-agarose gel. The size of the amplified part of the X88 gene and of competitor DNA is 0.99 kb and 0.59 kb, respectively. The point of equivalence in intensity of the competitor DNA and X88 bands designates the concentration of the X88 mRNA [10]. Four independent experiments with similar results were carried out. (B) Stage-specific expression of X88. Immunoblot analysis of 5 µg of lysates of Con A blasts (lane 1), unstressed TpM (lane 2), purified sporozoites (lane 3) and piroplasms (lane 4). Lysates were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to filters and probed with mAb TPB1 [17]. A *Eco*RI fragment of X88 comprising the first 1222 bp was subcloned in expression vector pGEX-1λT. Expression and purification of the GST-fusion protein was achieved as described [17]. This recombinant N-terminus of X88 was designated as GST:hsp 70 (A). Balb/c mice were immunised with purified GST:hsp 70 (A) using standard protocols. Spleen cells were fused to P3-X63-Ag8 myeloma cell line as described [18], the established hybridoma specific for GST:hsp 70 (A) was named TPB1 and produces antibodies of the IgG1, κ isotype. TpM and Con A blasts were cultivated [19] and the isolation of sporozoites and piroplasm was carried out as described [20].

Fig. 2.

parva has led to the isolation of cDNA clone X88 with significant homology to hsp 70 genes. Analysis of X88 nucleotide sequence revealed an open reading frame encoding a predicted polypeptide of 647 amino acid residues with a molecular mass of 71 kDa. The N-terminus contains a sequence GIDLGTTYSCV, which is present in all hsp 70 homologues identified so far [5]. All eucaryotic cytosolic hsp 70 molecules contain a sequence related to the consensus sequence GPTIEEVD at the extreme C-terminal end which is also present in X88 [5]. The deduced amino acid sequence of X88 clone displays an overall identity of 94.5, 97.7 and 72.1% to hsp 70 derived from *T. sergenti* [6], *T. annulata* [7] and bovine [8] respectively (Fig. 1 and data not shown).

Southern blot analysis of genomic DNA isolated from TpM suggests that the X88 encoded protein is likely to be either the product of a single or of few, tandemly arranged genes (data not shown). Hybridisation of X88 with *Sfi*I-digested *T. parva* genomic DNA resolved by pulse field gel electrophoresis localised the gene to the 1,200 kb *Sfi*I fragment 2 found on chromosome 1 of the genome (data not shown) [9]. The existence of other related hsp 70 genes cannot, however, be excluded since the filters were washed under high stringency conditions.

To determine whether X88 mRNA is inducible by heat stress we investigated the level of mRNA synthesis by TpM before and after heat shock treatment employing quantitative reverse transcriptase polymerase chain reaction (RT-PCR) [10]. It was evident that expression of X88 mRNA was upregulated in hsTpM compared with unstressed TpM (Fig. 2A). This result was confirmed by Northern blot analysis (data not shown). The upregulation of X88 expression by heat stress may have physiological significance since the transition from schizont to merozoite stage can be induced by incubating TpM at elevated temperatures [11]. It is instructive that cattle suffering from East Coast fever run temperatures as high as 42°C in the later stages of infection when merogony is in progress [1].

To analyse the expression and localisation of X88 protein at different stages of the parasite life cycle, mAb TPB1 was raised against its N-termi-

nus. Immunoblot analysis of ConA blasts, unstressed TpM, purified sporozoites and piroplasms with TPB1 revealed that X88 protein is expressed in all three life cycle stages (Fig. 2B). Hsp 70 expressed in unstressed cells are often referred to as 'cognate' hsp 70 [12]. Immunoelectron microscopy of TpM employing TPB1 revealed that the epitope recognised appears to be distributed uniformly throughout the cytosol of the schizont with no obvious association with the schizont membrane or host cytosol consistent with the consensus sequence in its C-terminus (data not shown) [5].

Hsp molecules are abundant and highly conserved among various micro-organisms, and in addition are major targets of the host immune response [4]. We have reported previously that hsp 70 proteins isolated from TpM are detected by Western blot analysis using sera derived from animals that have recovered from *T. parva* infection [13]. It has been demonstrated that hsp 70 can elicit specific cytotoxic immunity to tumors from which they are isolated [14]. Analysis of the basis of this immunity suggested that the antigenicity of hsp 70 preparations derives not from these stress proteins per se but from associated, co-purified peptides [15]. Since it is now possible to isolate parasite hsp 70 from hsp 70 proteins derived from TpM, we are currently examining whether the inoculation of cattle with parasite hsp 70 can induce a *T. parva* specific cytotoxic immune response.

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