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Short communication

Molecular characterization of the mitochondrial heat shock protein 60 gene from *Trypanosoma brucei*

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Stress proteins, including heat shock proteins 60 (hsp60s) and hps70s, are of considerable interest for the study of parasites for several reasons. First, the life cycle of most parasites involves the transfer of parasitic cells from an insect to a mammalian host, thereby subjecting the organism to a change in ambient temperature (heat shock). The heat shock proteins (hsp60, hsp70, etc.), the expression of which is increased in heat shock conditions, are thought to be involved in the differentiation process of the parasite [1]. Second, the stage-specific forms of many parasites exhibit structural and metabolic differences. For example, the mammalian forms of African trypanosomes lack the mitochondrial respiratory chain and the Krebs' cycle enzymes, while the mitochondria of the procyclic forms are fully active [2]. Considering the key position of the hsp60s and hsp70s in the import of proteins into the mitochondria, these chaperones could have a role in adaptation and regulation of mitochondrial functions. Third, during infection of the mammalian host with either intracellular or extracellular pathogens, including parasites, heat shock proteins are principal targets for the immune response, and could be considered as potential antigens for vaccine strategies [3,4]. We report in this paper the molecular cloning of a cDNA encoding the hsp60 from *Trypanosoma brucei*, and the use of a specific monoclonal antibody to study the expression of hsp60 in different trypanosomatids.

A cloned cDNA fragment (ptb60) containing an open reading frame encoding a peptide with a high

Abbeviation: hsp, heat shock protein.

Note: Nucleotide sequence data reported in this paper are available in the GenBank database under the accession number L43797.

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degree of similarity with other hsp60s, was randomly isolated from a cDNA library (data not shown). We used the ptb60 cDNA fragment as a probe to isolate cDNA clones from an oligo(dT)-primed cDNA library of *T. brucei* (EATRO-164). All the 11 cDNA fragments analyzed had the same nucleotide sequence and encoded a polypeptide of 562 amino acids with a predicted molecular mass

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Tb                                MFRCVVRFGAKDIRFGTEARQSMLKGVQRAVEAVATTLGPKGRNVIIEQSYGAPKITKDGVTVAKSIEF
Lt                                .L.SA..LAG..V...ED..R..Q...T...A.............
Tc                                ...SAA..AG.E..........Q.......S.................................A...
Y                    MLRSSVVRSRATLRPLLRRAYSSH.ELK..V.G.A.L....ETLA....A.........L...PF.P.............VL
H                 MLRLPTVFRQMRPVSRVLAPHLT.AY...VK..AD..AL..Q..DLLAD...V.M.....T......W.S..V...........DL
Ec                                MA...VK..ND..VK..R..NVLAD..KV.........VLDK.F...T......S..RE..L

Tb    KDPFENMGAQLVRQVCNKTNDLAGDGTTTSAVLVASIFSEGIKSIATGTNPIDMKRGMDRAVEVILKNIESQSRTVTNTENVVQVA
Tc    .....................................V...SLRC.................G...QSVAE...K..S...I....
Y     ..K......K.LQE.AS...EA......SAT..GRA..T.SV.NV.A.C..M.LR..SQV...KVIEFLSANKKEI.TS.EIA...
H     ..KYK.I..K..QD.A.N..EE.......AT..AR..AK..FEK.SK.A..VEIR..VML..DAVIAELKK..KP..TP.EIA...
Ec    E.K.......M.KE.AS.A..A....NN.AT..AQA.IT..L.AV.A.M..M.L...I.K..TAAVEELKAL.VPCSDSKAIA..G

Tb    TISANGDVELGKLIGEAMEKVGKDGVITTQDGKTLTTELEVVEGMSVDRGYISPYFVTDAKTQKAELEDAFVLVSAKKLNNIHTIL
Tc    .......E...R...Q.................M..........I.............A.................VSS.....
Y     .......SHV...LAS.......E....IRE.R..ED....T...RF...F.....I..P.SS.V.F.KPLL.L.E..ISS.QD..
H     .......KDI.NI.SD..K...RK....VK.....ND...II...KF.........INTS.G..C.FQ..Y..L.E..ISS.QS.V
Ec    ....TS.ETV....A...D....E....VE..TG.QD..D.....QF....L....INKPE.GAV...SP.I.LAD..IS..REM.

Tb    PVLNHVVRSGRPLLIIADDVESEALTTMIFNKLQGKLKIACVKAPGFGDNKAAMLQDIAIFSGACVVGEEGSGVELDAEKFEASIL
Tc    .A......T.....................................T..M......A..RL.......L.....N.DPA..
Y     .A.EISNQ.R.......E..DG...AAC.L...R.QV.VCA.........RKNTIG...VLT.GT.FT..-LD.KP--.QCTIEN.
H     .A.EIANAHRK..V...E..DG...S.LVL.R.KVG.QVVA.........RKNQ.K.M..AT.GAVF....LTLN.--.DVQPHD.
Ec    ...EA.AKA.K......E...G...A.AVV.TIR.IV.V.A........RRK.......TLT.GT.IS..-I.M..--.KATLED.

Tb    GSVKKATITKDDTVLLNGGGDVAMMKERVDLVRGLIERETSD-YNREKLQERLAKLSGGVAVIRVGGASEVEVNEKKDRITDALCS
Tc    .T.................ESS.V......L....DG....-.....................K...G.....................
Y     ..CDSI.V..E...I...S.PKEAIQ..IEQIK.S.DIT.TNS.EK.........................G.....YD...NA
H     .K.GEVIV....AM..K.K..K.QIEK.IQEIIEQLDVT..E-.EK...N.......D....LK...T.D.........V....NA
Ec    .QA.RVV.N..T.TIID.V.EE.AIQG..AQI.QQ..EA...-.D.......V...A......K..A.T...MK...A.VE...HA

Tb    TRAAVQEGIVPGGGAALLRASKALDGLLQDQSLTADQRTGVQIIRNAVRLPAHRIVANAGREGAVVVEKVLEN--TDAAVGYDAQL
Tc    .............V..........S..G.S.....................T..L...K.............--N.VT......R
Y     .....E...L....T..VK..RV..EVVVDNF---..KL..D...K.ITR..KQ.IE...E..S.IIG.LIDEYGD.F.K....SK
H     .....E....L...C....CIP...S.TPANE---..KI.IE..KRTLKI..MT.AK...V..SLI...I.Q---SSSEI....MA
Ec    .....E..V.A......I.VASK.AD.RGQNE---..NV.IKVALR.MEA.LRQ....C.E.PS..ANT.K---GG.GNY..N.AT

Tb    DRYVNMFEAGIIDPARVVRVALTDAASVASLMMTAEAAVVDLPKDDAP---AAGGMGGMGG-MGGMDGMY
Tc    ....................I...V..........I.....EET.---..........-....GD..
Y     SE.TD.LAT.....FK...SG.V..SG....LA.T.V.I..A.EPP.AA-G.-...P.GMPG.P..M
H     GDF...V.K.....TK...T..L...G....LT...VV.TEI....KD--PGM.A......G...GMF
Ec    EE.G..IDM..L..TK.T.S..QY.....G..I.T.CM.T....N..ADLG..........-....M
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Fig. 1. Comparison of the predicted amino acid sequence of the *T. brucei* hsp60 cDNA with other hsp60s. The protein encoded by the ptb60c cDNA (Tb) is compared with the hsp60 from *T. cruzi* (Tc) [10], yeast (Y) [11], the human P1 protein (H) [12] and the *E. coli* GroEL protein (Ec) [13]. The peptide encoded by the polymerase chain reaction fragment cloned from *Leishmania tarentolae* (Lt) is also included. The amino acids obtained by microsequencing the N-terminus of the mitochondrial hsp60 from *Leishmania tarentolae* are underlined. Gaps (–) are introduced to optimize the alignments, and the identities with the *T. brucei* hsp60 sequence are indicated with dots (.) into the five other sequences.

of about 63.7 kDa. A computer-assisted analysis of the deduced amino acid sequence revealed this protein (henceforth referred to as hsp60) to be a member of the 60 kDa family of heat shock proteins. The short 3′ non-translated region (247 bp) of the ptb60c cDNA contains two (A+T) motifs mainly composed of the TTA and TTTA repeats (data not shown). Such motifs have already been identified in genes encoding heat shock proteins from trypanosomes [5–7] and other eukaryotes [8], and are suspected to play a role in the heat-inducible expression of these genes [9].

A database search of the translated amino acid sequence of the ptb60c cDNA clone revealed that the *T. brucei* hsp60 is about 50% identical to most of the proteins belonging to the hsp60 family (50.4, 50.6 and 51.3% identity with the human P1 protein, the hsp60 form yeast and the GroEL protein from *E. coli*, respectively) (Fig. 1). Recently, a multigene family coding for a protein homologous to the hsp60 has been reported in *T. cruzi* [10,14]. Both trypanosomal hsp60s are 562 amino acids long. They are 87% identical and have 94.5% similarity when conservative changes are included (Fig. 1). This suggests that we have characterized the *T. brucei* hsp60 homologue of *T. cruzi*. The predicted amino acid sequence of the *T. brucei* hsp60 reveals a repeated GGM motif at the carboxyl terminus of the protein, which is conserved in all the members of the hsp60 family and is also found at the carboxy terminus of the cytosolic hsp70 from *T. brucei* [5] and *L. major* [15].

Northern blot analysis using an hsp60-specific probe showed the presence of a 2-kb messenger RNA which is more abundant in the procyclic forms than in the bloodstream forms of *T. brucei* (data not shown). To analyze the differential expression of the hsp60 genes at the protein level, polyclonal and monoclonal antibodies were raised against a recombinant hsp60 polypeptide which consists of the glutathione *S*-transferase protein fused with 279 amino acids of the trypanosomal hsp60 (position 94 aa to 372 aa). A monoclonal antibody H95, recognizing a single 60-kDa protein from *T. brucei*, was obtained and used for all further analysis. To compare the relative expression of the hsp60 in the procyclic and bloodstream forms of *T. brucei*, different amounts of cells from

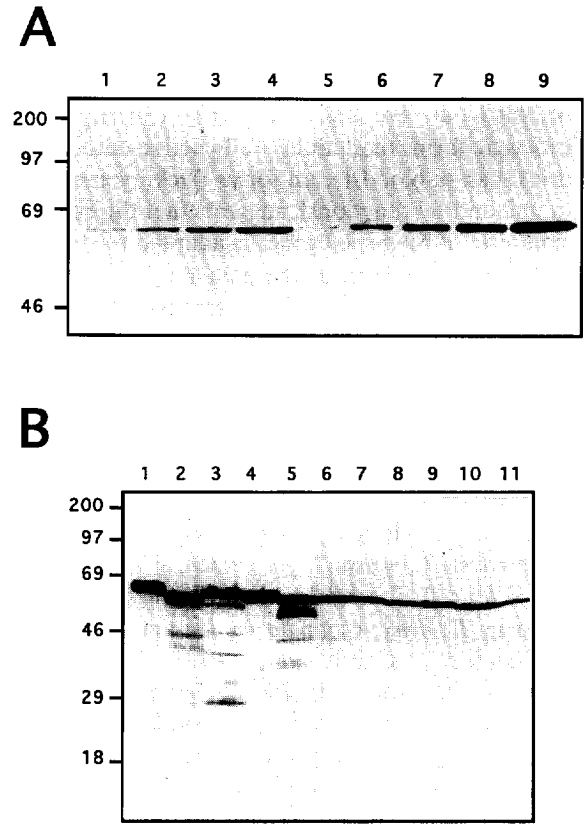

Fig. 2. Differential expression of hsp60 in *T. brucei* procyclic and bloodstream forms and detection in other trypanosomatids. (A) *T. brucei* lysate proteins were separated by electrophoresis, transferred onto a nylon membrane and probed with the H95 monoclonal antibody. Lanes 1–4 contain 1.25×10^6, 2.5×10^6, 5×10^6 and 10^7 trypomastigote forms, respectively; while lanes 5–9 contain 6×10^5, 1.25×10^6, 2.5×10^6, 5×10^6 and 10^7 procyclic forms, respectively. The data shown are from one out of three independent experiments which yielded similar results. (B) Lysates of 10^7 cells from *C. lucilae* (lane 1), *T. cruzi* (lane 2), *L. major* (lane 3), *T. vivax* (lane 4), *T. congolense* (lane 5), *T. equiperdum* BoTat1 (lane 6) and BoTat28 (lane 7), *T. evansi* (lane 8), *T. rhodesiense* (lane 9), *T. gambiense* (lane 10) and *T. brucei* (lane 11) were processed for Western blotting as indicated in panel A. The positions of molecular size standards (in kDa) is shown in the left margin of both panels.

both stage-specific forms were analyzed by western blotting. The results show that the hsp60 is 2–4 times more abundant in the procyclic forms than in the bloodstream forms (Fig. 2a). The differential expression of the hsp60 gene(s) is also reflected at the mRNA level (data not shown), suggesting a

regulation at the transcriptional or post-transcriptional level, while a moderate additional translational or post-translational control can not be ruled out. The higher expression of hsp60 in procyclic forms (containing fully active mitochondria) than in bloodstream forms (lacking essential mitochondrial functions), suggests that the expression level of the hsp60 genes is adapted to the mitochondrial function of the parasite.

Expression of the hsp60 was investigated in other members of the trypanosomatids and in a dyskinetoplastic *T. equiperdum* clone BoTat28 [16] by use of the H95 monoclonal antibody. A 60–65-kDa protein, constituting the strongest signal is detected in all the parasites analyzed so far (Fig. 2b). The apparent size of the hsp60 is the same for the parasites belonging to the *T. brucei* group (*T. equiperdum*, *T. evansi*, *T. gambiense*, *T. rhodesiense* and *T. brucei*), *T. congolense* and *T. cruzi* (60 kDa), while in *T. vivax*, *Crithidia lucilae* and *L. major*, this protein is slightly larger (61–65 kDa). This suggests that some significant differences exist among the hsp60s from trypanosomatids. In addition, the hsp60 protein was also identified in dyskinetoplastic *T. equiperdum* (BoTat28) [16], showing that the protein is encoded in the nucleus.

Sullivan et al. showed that the hsp60 from *T. cruzi* is located in the mitochondrial matrix [14]. Unfortunately, the polyclonal antibodies raised against the glutathione *S*-transferase fusion protein gave negative results when tested by immunofluorescence against *T. brucei*. Therefore we tested the localisation of hsp60 on a well characterized mitochondrial fraction of *Leishmania tarentolae* [17,18]. Western blot analyses using the H95 monoclonal antibody revealed that the hsp60 is highly enriched in that fraction (data not shown) [19]. To determine the N-terminus extremity of the hsp60, a mitochondrial fraction of *Leishmania tarentolae* was run into a two-dimensional native/SDS-polyacrylamide gel [19], blotted onto Immobilon-P^{SQ} filters (Millipore) [20] and the 62-kDa protein immunodetected by the H95 monoclonal antibody was microsequenced (data not shown). The N-terminal sequence analysis revealed a sequence of 10 residues similar to the sequence starting at position 9 of the hsp60-encoded gene from *T. brucei* and *T. cruzi*. To confirm this result, the 5′ portion of the cDNA encoding the hsp60 from *L. tarentolae* was polymerase chain reaction-amplified using a 3′ degenerate oligonucleotide derived from back-translation of the *T. brucei* and *T. cruzi* hsp60 amino acid sequence (GPKGRNVIIE), and a 5′ oligonucleotide derived from the 39-nt miniexon sequence present at the 5′ end of all trypanosomatid mRNAs [21] (data not shown). As expected, the N-terminal sequence of the leishmanial protein lacks the first eight residues predicted from the *L. tarentolae* hsp60 genes (Fig. 1) which may constitute a cleavable mitochondrial import signal. The nature of these eight amino acids is consistent with a mitochondrial import signal function (richness in basic, hydroxylated and hydrophobic amino acids), and the N-termini of the encoded hsp60 from *L. tarentolae*, *T. brucei* and *T. cruzi* are seven amino acids longer than the N-terminus of GroEL from *E. coli* and eight amino acids longer than the mature human P1 protein (Fig. 1). Furthermore, the size of this predicted mitochondrial import signal is close to the size of cleavable N-terminal putative mitochondrial import signals already described in trypanosomes. Among the seven described trypanosomal putative import signals, five are nine amino acids long. These consist of three proteins associated with the kDNA from *C. fasciculata* [22], the dihydrolipoamide dehydrogenase from *T. brucei* [23] and the mitochondrial aldehyde dehydrogenase from *L. tarentolae* [19]. Two trypanosome mitochondrial signal sequences are longer than nine amino acids: 17 amino acids for the mitochondrial hsp70 from *C. fasciculata* [24] and *L. major* [25] and 20 amino acids for an 18-kDa putative gRNA binding protein from *L. tarentolae* [19].

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