Identification of potential HLA class I and class II epitope precursors associated with heat shock protein 70 (HSPA)

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Received: 27 October 2009 / Revised: 20 February 2010 / Accepted: 23 February 2010 / Published online: 1 April 2010
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Abstract Heat shock protein 70 (HSPA) is a molecular chaperone which has been suggested to shuttle human leukocyte antigen (HLA) epitope precursors from the proteasome to the transporter associated with antigen processing. Despite the reported observations that peptides chaperoned by HSPA are an effective source of antigens for cross-priming, little is known about the peptides involved in the process. In this study, we investigated the possible involvement of HSPA in HLA class I or class II antigen presentation and analysed the antigenic potential of the associated peptides. HSPA was purified from CCRF-CEM and K562 cell lines, and using mass spectrometry techniques, we identified 44 different peptides which were co-purified with HSPA. The affinity of the identified peptides to two HSPA isoforms, HSPA1A and HSPA8, was

confirmed using a peptide array. Four of the HSPA-associated peptides were matched with 13 previously reported HLA epitopes. Of these 13 peptides, nine were HLA class I and four were HLA class II epitopes. These results demonstrate the association of HSPA with HLA class I and class II epitopes, therefore providing further evidence for the involvement of HSPA in the antigen presentation process.

Keywords Heat shock protein 70 (HSPA) · Human leukocyte antigen (HLA) · Antigen presentation · Molecular chaperone

Introduction

The first reports of an immunological role for heat shock proteins (HSPs) were published over two decades ago (Srivastava et al. 1986; Ullrich et al. 1986) where it was shown that isolated HSPs when used as an immunogen protected syngeneic mice from the same tumour challenge and induced tumour-specific immunity. Since then, it has been reported that HSPs, including heat shock protein 70 (HSPA), were effective in cross-priming CD8⁺ T cells against the tumour from which these chaperones were isolated, recently reviewed by Srivastava (2002). This led to the hypothesis that HSPA is chaperoning HLA class I epitopes during their generation (Binder et al. 2001). Antigen presentation includes HLA class I and class II and is an essential part of adaptive immunity and immunological tolerance. HLA class I is expressed on almost all nucleated cells, and in most cases, its epitopes are of endogenous origin. CD8⁺ T cells specifically recognise HLA class I antigens and eliminate virally infected cells or tumour cells. HLA class II molecules are exclusively

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expressed on antigen-presenting cells (APCs) which also express HLA class I molecules. Peptides presented on HLA class I and class II molecules by APCs are of endogenous and exogenous origin. Antigens presented by APCs on HLA class II are recognised by CD4⁺ T cells which regulate adaptive immunity (Jensen 2007).

Endogenous HLA class I epitopes are the products of proteasomal degradation and experience a multistep generation process. In most cases, the proteasome produces peptides with correct C-termini for HLA class I epitopes (Cascio et al. 2001). However, the generation of correct C-termini has also been attributed to tripeptidyl peptidase II (TPPII) which has been shown to be essential for producing particular epitopes (Geier et al. 1999; Seifert et al. 2003). Peptides from the cytosol are transported by the transporter associated with antigen processing (TAP) to the endoplasmic reticulum (ER) (Kleijmeer et al. 1992) where their N-termini are specifically trimmed, in the final step of antigen generation, to a particular size of eight or nine residues by ER-associated aminopeptidases (Saveanu et al. 2005; York et al. 2002). HLA class II, in contrast, is specifically expressed by APCs which take up exogenous antigen precursors and transport them to the MHC class II compartment (MIIC) where antigen generation takes place (Neefjes et al. 1990).

In order for CD8⁺ T cells to become competent effector cells, they have to be primed by APCs against a specific endogenous or exogenous antigen. Cross-priming is a crucial pathway of exogenous antigen presentation on the HLA class I molecules by APCs, enabling antiviral or anti-tumour responses (den Haan et al. 2000; Sigal et al. 1999). It has been shown that intact proteins or non-chaperoned proteasome products can be a source of antigens used for cross-presentation (Norbury et al. 2004; Serna et al. 2003; Shen and Rock 2004). However, these molecules were shown not to be as effective in cross-priming as peptides chaperoned by HSPs (Binder et al. 2001; Binder and Srivastava 2005). Consequently, it has been suggested that particular HSPs are involved in the antigen presentation process. The endogenous and exogenous (TAP-dependent) antigen generation for HLA class I presentation, as well as the generation of endogenous HLA class II epitopes, depends on the proteasome (Kovacsóvics-Bankowski and Rock 1995; Mukherjee et al. 2001; Norbury et al. 2004; Tewari et al. 2005). Peptides produced by the proteasome are prone to rapid degradation in the cell (Fruci et al. 2003; Princiotta et al. 2003). Therefore, the role of the cytosolic HSPs may be to prevent this degradation and thus make the peptides available for HLA class I and class II presentation.

In this study, we have isolated peptides associated with HSPA from two human leukemic cell lines, K562 and CCRF-CEM. The purified cytosolic HSPA contained the two isotypes, HSPA1A and HSPA8. Of the 44 peptides

identified, four peptides were found to be precursors for 13 previously reported HLA class I and class II epitopes. Therefore, our results demonstrate further that HSPA takes part in the antigen presentation process by securing the epitope library for HLA class I and class II presentation.

Experimental

Cell culture K562 and CCRF-CEM leukemic cell lines were grown in spinner bottles in RPMI 1640 culture medium (Gibco) containing 100 IU/ml penicillin, 100 µg/ml streptomycin (Gibco), and 2 mM L-glutamine (Gibco) supplemented with 10% heat-inactivated foetal calf serum (Sera Lab). The cell lines were incubated at 37°C in a humidified 5% CO₂ air incubator. The cells were grown in continuous culture and were harvested when they reached confluence, washed in Dulbecco's PBS (Lonza) and stored at -80°C. Approximately 10 g of cell pellet was collected for each cell line, corresponding ~6×10⁹ and ~1.2×10¹⁰ cells for K562 and CCRF-CEM, respectively.

Protein purification and peptide isolation The cells were thawed, resuspended in a homogenisation buffer (10 mM Tris-HCl, pH 7.6, 0.5 mM MgCl₂) containing protease inhibitors (complete EDTA-free, Roche), and incubated for 30 min at room temperature, after which they were frozen and thawed before Dounce homogenisation at 4°C (cell disruption was examined microscopically). HSPA purification was performed according to the method of Peng et al. (1997) with modifications. In brief, the cell lysate was centrifuged at 100,000×g for 1 h and the supernatant collected. The supernatant was adjusted to 50 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 5 mM MgCl₂ and applied overnight onto a 7 ml adenosine diphosphate (ADP) agarose column (Sigma-Aldrich) at 4°C. The column was then washed with 50 mM NaCl, 10 mM Tris-HCl, pH 7.6, and 5 mM MgCl₂. Finally, elution from the column was performed using the same buffer containing 3 mM ADP (Sigma-Aldrich). Fractions containing HSPA were pooled and subjected to buffer exchange on Sephadex™ G-25 (Amersham Biosciences) to 30 mM ammonium hydrogen carbonate (Sigma-Aldrich) in ultrapure water (Sigma-Aldrich). The samples were freeze-dried three times in a glass round bottom flask and each time resuspended in 10 ml of ultrapure water.

The method for HSPA purification has been previously described and shown to isolate the protein with associated peptides (Peng et al. 1997); however, the method to isolate the associated peptides from HSPA had to be developed. A cell lysate was used to determine optimal conditions for size separation on centricon 10 (Millipore). Therefore, a cell lysate either in PBS only, PBS with 10 mM ATP or

0.1% trifluoroacetic acid (TFA) and 25% acetonitrile (ACN) was loaded onto centricon 10 (Millipore) with a cutoff size of 10 kDa and spun. Centricon was observed to be pervious to low-molecular-weight proteins and peptides only in the presence of TFA/ACN as analysed using tricine sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE, data not shown). Consequently, TFA/ACN was used to denature HSPA, releasing the associated peptides which were then size-filtered through centricon. The flow-through was collected, freeze-dried and resuspended in 400 µl of 0.1% TFA.

Western blotting The detection of HSPA on Western blots was performed with primary antibodies: anti-HSPA1A (Assay Designs, SPA-810), anti-HSPA8 (Assay Designs, SPA-816) or anti-HSPA (Abcam, ab5439) and horseradish peroxidase (HRP)-conjugated secondary antibodies: either anti-mouse (Sigma-Aldrich, A5278) or anti-rabbit (Sigma-Aldrich, A0545). For visualisation, ECL Plus detection reagents (GE Healthcare) were used. Densitometry analysis was performed using Quality One software version 4.9.5 (Bio-Rad).

HPLC separation and mass spectrometry Peptides in 0.1% TFA were concentrated and desalted with a C18-ZipTip (Millipore) and eluted with 0.1% TFA, 50% ACN before drying in a speed-vac. Peptides were resuspended in 0.1% formic acid solution (5 µl) and loaded onto a 0.3×5 mm Zorbax 300SB-C18 trap column (Agilent Technologies) which was switched in-line with a 0.075×100 mm Integra-Frit ProteoPep 2 column (New Objective) flowing at 200 nl/min. The gradient conditions were: 5% to 30% ACN in 35 min, followed by 30% to 50% ACN in 15 min. The ACN buffer was in 0.1% aqueous formic acid. Peptides eluting from the capillary column were analysed by nano-LC/MS in positive ion mode on a Finnigan LTQ-FT mass spectrometer (ThermoElectron) equipped with the Finnigan Nanospray ion source (ThermoElectron). Eluate was sprayed using uncoated 10 µm I. D. SilicaTips (New Objective) at a spray voltage of 2 kV. The mass spectrometer performed survey MS scans over the mass range $m/z=350$ – 1350 in data-dependent mode. MS data were acquired with a FT-MS resolution setting of 100,000 (at $m/z=400$), and the four top ions in the parent scan were automatically subjected to MS/MS in the linear ion trap region of the instrument. Base peak mass spectrum plots and MS/MS fragmentation data were visualised using the QualBrowser programme (ThermoElectron).

Mass spectrometric data analysis To generate the peak lists, the BioWork software version 3.3 (ThermoElectron) with default parameters was used. To avoid false positive sequencing results, the following workflow was estab-

lished. The fragmentation data were independently analysed by two database search engines, Mascot (Perkins et al. 1999) and Sequest (Eng et al. 1994), and the deduced sequences proposed by the engines were combined and common peptide sequences were further manually de novo analysed. This allowed verifying the peptide sequences and provided a list of positively validated peptides. These peptides were searched against the two databases: SYFPEITHI (www.syfpeithi.de) and the Immune Epitope Database and Analysis Resource (IEDB; www.immuneepitope.org; Peters et al. 2005), which contain reported HLA class I and class II epitopes. The sequences of potential HLA class I epitopes were assessed using the binding motifs shown in SYFPEITHI.

Peptide affinity assay A peptide array of the HSPA-associated peptides was commercially manufactured (JPT Peptide Technologies). Due to the SPOT synthesis method restrictions, peptides which were longer than 20 residues were truncated to the length of 20 residues containing at least 15 residues of overlapping fragments (see Table 2). Therefore, peptides which were longer than 20 amino acids had multiple spots on the peptide array. Peptides were synthesised at 5 nmol per spot with an acetylated N-terminal end and were covalently bound by the C-terminal end with a polyethylene glycol linker to the cellulose membrane. Two isoforms of HSPA were analysed in an affinity assay: recombinant HSPA1A (Assay Designs, NSP-555) and recombinant HSPA8 (Assay Designs, SPP-751). The peptide array was blocked with 3% skimmed milk powder in TBS (150 mM NaCl, 10 mM Tris-HCl, pH 7.6) for 3 h, then incubated with 100nM HSPA1A/HSPA8 in TBS overnight at 4°C. The binding of the protein to the peptide array was detected with specific anti-HSPA primary antibodies (1-h incubation; Abcam, ab5439) 1:1,000 and HRP-conjugated secondary antibodies (1-h incubation; Sigma-Aldrich, A5278) 1:5,000. After each incubation step, the membrane was washed three times for 10 min with TBS containing 0.2% Tween 20. The negative control was performed with a combination of both primary and secondary antibodies only. Finally, antibody binding was visualised using ECL Plus detection reagents (Amersham Biosciences). A trans-luminescent scanner was calibrated with a photographic step tablet (Kodak) and the signal intensity was assessed. The picture was black to white inverted and the intensities of the spots were analysed with ProScan software (PerkinElmer). The peptides with either an intensity lower than the double average value of the negative control spots, or did not reach an intensity higher than a twofold increase over the corresponding control, were assessed as none binding. All the others were assessed as either moderate (when an intensity of higher than a twofold increase over the corresponding control was reached) or high-affinity binders (when an

intensity of higher than a fourfold increase over the corresponding control was reached).

Results

The biochemical characteristics of the peptides associated with HSPA

HSPA was purified from two leukemic cell lines, K562 and CCRF-CEM, using an affinity ADP column (Peng et al. 1997). The purification products were analysed by SDS-PAGE gel electrophoresis followed by silver staining and Western blotting (Fig. 1). On a silver-stained gel, the purified protein was shown to produce a double band at 72 kDa (Fig. 1a), which was verified by Western blotting to be HSPA8 and HSPA1A (Fig. 1b). We used a commercially available, purified HSPA1A as a control for the Western blotting which was shown to be recognised by anti-HSPA1A and anti-HSPA (it recognises several members of HSPA family), but not by anti-HSPA8 antibodies (Fig. 1b). Purified HSPA from K562 and CCRF-CEM were shown to be positive for HSPA1A and HSPA8 (Fig. 1b). K562 and CCRF-CEM purification products appeared as a double band when analysed with anti-HSPA antibodies (Fig. 1b), where the lower band localised at the same position as the control HSPA1A hence was recognised as HSPA1A; consequently, the upper band was assigned to HSPA8 (Fig. 1b). The silver-stained gel densitometry analysis showed the purity of HSPA from either

K562 or CCRF-CEM to be of over 75%. The densitometry examinations of the Western blotting developed with anti-HSPA (Fig. 1b) revealed that HSPA1A was approximately fourfold more abundant than HSPA8 in K562 and CCRF-CEM purification products.

HSPA-associated peptides were released from the protein using TFA/ACN solution and size-filtered on centricon 10. The flow-through fraction was applied onto a reverse phase

Fig. 1 Purified HSPA from K562 and CCRF-CEM used for the associated peptide analysis. The purification products were analysed by SDS-PAGE followed by silver staining (a) and Western blotting (b). Commercially available recombinant HSPA1A was used as a control for Western blotting. Three types of antibodies were used for HSPA detection: specific anti-HSPA1A, specific anti-HSPA8 and anti-HSPA that recognises several HSPA family members including HSPA1A and HSPA8. Anti-HSPA antibodies were shown to produce double bands. The lower band was localised at the same position as the control HSPA1A and was therefore identified as HSPA1A; the upper band was consequently associated with HSPA8

Fig. 2 Reverse phase HPLC elution profile and biochemical properties of the identified peptides co-purified with HSPA. Peptides were eluted from a C18 reverse phase HPLC column with an ACN gradient, 5–30% in 35 min and 30–50% in the following 15 min. **a** Reverse phase HPLC elution profile of peptides co-purified with HSPA from K562 and CCRF-CEM cell lines. **b** Distribution of the positively validated HSPA-associated peptides from K562 and CCRF-CEM cell lines overlaid onto the reverse phase HPLC elution profile. The ACN concentration required for the peptide desorption approximately correlates with its hydrophobic characteristic. **c** Residual length distribution of the HSPA-associated peptides

HPLC with elution over a 5–30% ACN gradient for 35 min followed by 30–50% over 15 min (Fig. 2a). The eluent was analysed using a Thermo Finnigan LTQ-FT mass spectrometer, and the raw fragmentation data were subjected to database searches performed by two independent search engines, Mascot (Perkins et al. 1999) and Sequest (Eng et al. 1994). Mascot identified 342 and 361 peptides, whereas Sequest deduced 813 and 830 peptide sequences for K562 and CCRF-CEM, respectively. These data from Mascot and Sequest were then cross-referenced, resulting in the identification of 28 and 25 common sequences for K562 and CCRF-CEM, correspondingly. The common peptide sequences deduced by the engines were further manually validated by de novo sequencing (Fig. 3); this approach eventually allowed the identification of 26 peptides from K562 cell line and 18 from CCRF-CEM.

Positively validated peptide sequences were overlaid onto an ACN gradient elution curve (Fig. 2b). Most of the successfully sequenced peptides were eluted between 25 and 35 min after the start of the gradient, and this correlated with 23% to 30% ACN. The HSPA-associated peptide elution time suggests a similar hydrophobic character for the peptides among the cell lines (Fig. 2b). The mass range of the HSPA bound peptides was 1078–3,210 Da and 9–33 amino acids in length (Fig. 2c). The average length of the HSPA-associated peptides was 17 and 15 amino acids for K562 and CCRF-CEM cell lines, respectively (Fig. 2c). The length distribution of the HSPA-associated peptides showed that only four peptides complied with the HLA class I epitope size restriction of

eight to ten amino acids (Rammensee et al. 1993). Peptides isolated with greater than ten residues suggests a need for further processing to meet the HLA class I epitope size restriction.

The number of peptides positively validated in this study was 44, and these were found to originate from 26 different proteins (Table 1). The diversity of the proteins from which the HSPA-associated peptides were derived was much greater for the K562 cell line compared to the CCRF-CEM cell line where 11 peptides, C1–C11, originated from glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The C6 peptide represents GAPDH protein and its sequence was common to another five peptides C1–C5. Twenty-five per cent (11/44) of the peptides were of the C-terminal end of the proteins from which they were derived (Table 1).

Analysis of the peptides also revealed an arrangement of hydrophobic residues (F, I, L, V; Hb) that were interspersed by hydrophilic ones (D, E, R, H, K, Q, N; Hp; Table 1; Kyte and Doolittle 1982). At least one pair of Hb-Hb or Hp-Hp residues was present in 82% of the peptides. At least one mixed pair, Hb-Hp or Hp-Hb, was common to 86% and 84% of the peptides, respectively. Hb-Hp-Hb segments were in 43% of the peptides and Hp-Hb-Hp in 32%. The arrangement of Hb-Hb-X-Hb (X—any amino acid) was common to 43% of all the HSPA-associated peptides (Table 1). The HSPA-associated peptides shown in this report partially matched to the hydrophobic characteristics described previously for peptides binding to HSPA (Fourie et al. 1994; Grossmann et al. 2004; Maeda et al. 2007; Takenaka et al. 1995).

Two pairs of peptide fragments were found to be consistent between the two cell lines, K9=C12 and K13=C11. K9=C12 represented GAPDH protein and shared 17 amino acids in common; K13=C11 shared a fragment of 15 amino acids derived from tubulin α 6 (Table 1). Thirteen peptides shared fragments of at least seven (K14, K15, K20, C1–C3, C9, C10, C11) or at least four (K13, C4–C6) residues with peptides previously reported to be eluted from HSPA purified from a mouse TRAMP C2 cell line (Grossmann et al. 2004; Table 1). These findings suggest a consistency in selection of peptides by HSPA.

Affinity study of the HSPA-associated peptides to HSPA

To test the ability of the identified peptides (Table 1) to bind HSPA, a peptide array was produced. Two members of the HSPA family, HSPA1A and HSPA8, were tested for binding to the peptides (Fig. 4). Due to technical limitations of the array production method, peptides longer than 20 amino acids had to be fragmented. Eight peptides, K8, K9, K11, K21, K26 and C1–C3, were truncated to 20 amino acid fragments, with at least 15 amino acids of overlapping fragments giving rise to 54 peptides in total which were

Fig. 3 Determination of the peptide sequence by de novo sequencing. The fragmentation spectrum of the peptide C10 (see Table 1) co-purified with HSPA from the CCRF-CEM cell line. *y*+ C-terminal fragmentation ions, *b*+ N-terminal fragmentation ions, *asterisk* mass of the fragment correlates with a dimer, SE or ES which was verified by NCBI BLAST, *m/z* mass-to-charge ratio of the ions. Differences between peaks correlate with a mass loss which represents a particular amino acid. *SEGPLKGILGY* the C10 peptide sequence deduced by de novo sequencing

Table 1 Human HSPA-associated peptides isolated from the K562 and CCRF-CEM cell lines

Peptide name ^a	MH ^b	Peptide sequence ^c	Protein (gi) ^d	Position ^e
K1	1295.79	<u>RKALDVITIAVP</u>	ATPase type 13A4 (66932949)	432-443/1196
K2	1889.00	<u>KSADTILWDIOKDLKDL</u>	lactatedehydrogenase B (4557032)	319-334/334
K3	2128.15	<u>VERMILKIDDIRKPGESFE</u>	chaperonin containing TCP1, subunit 5 (24307939)	524-541/541
K4	2030.13	<u>ATETVRSILKIDDVVNTR</u>	chaperonin containing TCP1, subunit 4 (38455427)	522-539/539
K5	1959.09	<u>TETVRSILKIDDVVNTR</u>	chaperonin containing TCP1, subunit 4 (38455427)	523-539/539
K6	1985.08	<u>PAVLGFEGSANKIGVGVRD</u>	O-sialoglycoproteinendopeptidase (8923380)	2-21/335
K7	1078.61	<u>LIHVPOKED</u>	inter-alpha inhibitor H1 (4504781)	676-684/911
K8	2549.28	<u>GGQSVYHVLHVLGGROMHWPPG</u>	histidine triad protein (4885413)	104-126/126
K9	2448.35	<u>AGKHVPRAVFDLEPTVIDEVR</u>	tubulin, alpha 6 (14389309)	58-79/449
K10	1684.93	<u>NSKILVWVTAGVROQEG</u>	lactatedehydrogenase B (4557032)	89-104/334
K11	2180.23	<u>DGRGGGPRSTAGGVALAVVVLSLA</u>	mannosyl-oligosaccharide glucosidase (116242490)	31-54/837
K12	1946.93	<u>FHPFQLITGKEDAAANNY</u>	tubulin, alpha 6 (14389309)	87-103/449
K13*	1922.82	<u>GYTEHQVVSDFNSDTH</u>	GAPDH (7669492)	275-291/335
K14**	1626.93	<u>YGKVIPELNGKLTGMA</u>	GAPDH (7669492)	217-232/335
K15**	2112.17	<u>RYAPEEHPITLITEAPLNPK</u>	actin, alpha 1 precursor (4501881)	97-115/377
K16	1735.80	<u>VSQEFMEFIQGGPE</u>	mitochondrial ribosomal protein S36 (15150811)	89-103/103
K17	1816.93	<u>SVVPSPKVSDTFVVEPVN</u>	tubulin, beta (29788785)	168-184/444
K18	1119.67	<u>KDILPRHIQ</u>	asparagine-linked glycosylation 6 (38026892)	289-297/507
K19	1396.78	<u>NILRASHSAVDIT</u>	gephyrin isoform 2 (66932975)	298-310/736
K20**	1954.06	<u>YAPEEHPVILLITEAPLNPK</u>	actin, beta (4501885)	96-113/375
K21	2530.37	<u>EENINLFIKKVKNFLLEENVLP</u>	laminin, beta 4 (143770880)	1475-1495/1761
K22	1575.92	<u>YPIIDPKLLEDLVQP</u>	neuronal cell adhesion molecule (81158226)	32-45/1304
K23	1911.96	<u>SEAFREANLAASFGKSLN</u>	dihydroipoamidedehydrogenase (91199540)	491-508/509
K24	2012.03	<u>ISKIREEYPDRIMNTF</u>	tubulin, beta (29788785)	152-167/444
K25	2125.97	<u>PPNPEEVKTPPEEGEKQSQC</u>	G antigen 8 (7705379)	98-116/116
K26	3211.39	<u>YDFEASAPGSGNPCHASAAOKENAGEDPGILA</u>	dermcidinpreproprotein (16751921)	20-52/110
C1**	2962.34	<u>ISWYDNEFGYSNRVVDLMAHMASKE</u>	GAPDH (7669492)	311-335/335
C2**	2576.15	<u>YDNEFGYSNRVVDLMAHMASKE</u>	GAPDH (7669492)	314-335/335
C3**	2413.09	<u>DNEFGYSNRVVDLMAHMASKE</u>	GAPDH (7669492)	315-335/335
C4*	2076.97	<u>ISWYDNEFGYSNRVVDL</u>	GAPDH (7669492)	311-327/335
C5*	1658.75	<u>DNEFGYSNRVVDLM</u>	GAPDH (7669492)	315-328/335
C6*	1527.71	<u>DNEFGYSNRVVDL</u>	GAPDH (7669492)	315-327/335
C7	1540.81	<u>DAGAGIALNDHFVKL</u>	GAPDH (7669492)	296-310/335
C8	1425.78	<u>AGAGIALNDHFVKL</u>	GAPDH (7669492)	297-310/335
C9**	1776.93	<u>GMAFRVPTANYSVVDLT</u>	GAPDH (7669492)	230-246/335
C10**	1133.62	<u>SEGPIKGIIGY</u>	GAPDH (7669492)	266-276/335
C11**	2206.00	<u>GILGYTEHQVVSDFNSDTH</u>	GAPDH (7669492)	272-291/335
C12	1701.91	<u>AVFVDLEPTVIDEVR</u>	Tubulin, alpha 6 (14389309)	65-79/449
C13	1178.56	<u>WWYAYNDKP</u>	Coiled-coil domain containing 141 (39725717)	867-875/875
C14	1333.74	<u>VSSTINPIILYNL</u>	Neurotensin receptor 1 (110611243)	355-366/418
C15	1492.77	<u>MKVEVLPAITDNY</u>	Hydroxyacyl glutathione hydrolase (94538320)	1-13/260
C16	1746.88	<u>AGFAGDDAPRAVFPSTVG</u>	Actin, alpha 1 precursor (4501881)	21-38/377
C17	1710.68	<u>MYEGLWMSCVQSSTG</u>	Claudin 1 (10863887)	46-60/211
C18	1179.57	<u>DKLKGEMMDL</u>	Lactatedehydrogenase A (5031857)	56-65/332

^a Index name, K and C indicate the cell line from which the HSPA-associated peptide was eluted, K562 and CCRF-CEM, respectively. Peptides which share a common fragment of at least four (*) and (**) seven residues with the HSPA-associated peptides isolated from a mouse TRAMP C2 cell line (Grossmann et al. 2004)

^b Measured mass of the monoprotonated peptide ion

^c Sequence of the HSPA-associated peptide. *Dotted underline*, hydrophobic amino acids (F, I, L, V); *solid underline*, hydrophilic amino acids (D, E, R, H, K, Q, N). In *bold*, peptide fragments common to the HSPA-associated peptides isolated from a mouse TRAMP C2 cell line (Grossmann et al. 2004)

^d Protein name from which the peptide was retrieved; (gi) gene identifier number

^e Position of the peptide and a total number of amino acids in the protein sequence

Fig. 4 Relative binding affinity of recombinant HSPA to the HSPA-associated peptides. The peptides were synthesised on a membrane by JPT Peptides Technologies. Peptides longer than 20 amino acids were truncated to 20 amino acid fragments with at least 15 amino acids of overlapping fragments. Thus, peptides longer than 20 residues had multiple spots on the peptide array. Two isoforms of HSPA were tested: HSPA1A and HSPA8. HSPA1A or HSPA8 (100 nM) was incubated with the peptide array overnight at 4°C, detected with specific primary antibodies and visualised with HRP-conjugated secondary antibodies. Incubating the peptide array with both primary and secondary antibodies but without either HSPA1A or HSPA8 was performed as a negative control. The relative affinity of the peptides to HSPA1A and HSPA8 was analysed and presented in Table 2

synthesised on the peptide array (Fig. 4 and Table 2). The affinity of HSPA1A/HSPA8 to the peptides was detected with specific primary antibodies and visualised with HRP-conjugated secondary antibodies (Fig. 4). The control experiment was performed by incubating the peptide array with a combination of both primary and secondary anti-

bodies, which showed no specific binding to the peptides (Fig. 4). The relative affinity was obtained by analysing the intensity of the spots (Table 2). Forty-four out of 54 peptides on the array were recognised by at least one of the tested isoform of HSPA, with 34 of 44 peptides showing high binding affinity (Fig. 4 and Table 2). Nineteen peptides on the array were defined as none binding by either one of the isoforms, and ten peptides were assessed as none binding by both of the isoforms (Fig. 4 and Table 2). The truncated peptides, derived from K8, K9, K11, K21, K26 and C1–C3, in general presented very similar affinity to the proteins (Fig. 4).

The HSPA-associated peptides assessed for the presence of HLA epitopes

We analysed the HSPA-associated peptides isolated from the CCRF-CEM and K562 cell lines for their suitability to be presented on HLA class I. CCRF-CEM is positive for HLA class I (Liu et al. 1999), whereas K562 can only be induced to express HLA class I and class II when stimulated with interferon- γ (Ress et al. 1991). The pool of the CCRF-CEM HSPA eluted peptides shown in Table 1 was subjected to HLA class I epitope database searches with the restrictions to the HLA alleles of the cell line. The leukemic cell line, CCRF-CEM, has been reported to express HLA class I alleles, including A*0101, 31012; B*0801, 4001 and C*0304, 0701 (Adams et al. 2005; Masuda et al. 2007). As shown in Fig. 2c and Table 1, 89% of the CCRF-CEM HSPA-associated peptides had too many residues to meet HLA class I basic length requirements (Rammensee et al. 1993). Therefore, the peptides were treated as potential precursors for HLA class I epitopes which would need to be subsequently truncated. Regarding the HLA type of the CCRF-CEM cell line and relying on HLA class I binding motifs listed in the SYFPEITHI database, we propose seven potential epitopes which could possibly be presented on HLA class I (Table 3). Although these potential epitopes at least partially met the anchoring restrictions, none of them had previously been reported in the SYFPEITHI or the IEDB databases as eluted from HLA class I.

To investigate if HSPA in general is able to bind HLA epitopes, the HSPA-associated peptides isolated from K562 and CCRF-CEM cell lines were searched against the HLA class I epitope databases regardless of HLA type. Again, truncation of the peptides was allowed to take into account HLA class I size restrictions. Amongst four (K15, K20, K24 and K26) of the HSPA-associated peptides, nine reported HLA class I epitopes were matched; three peptide fragments, 2x K20-B and 1x K24-A, were exact matches to the reported epitopes, and six peptide fragments, 2x K15-B, 1x K15-A, 1x K20-A, 1x K24-B and 1x K26-A, contained

Table 2 Relative binding affinity of recombinant HSPA to the HSPA-associated peptides

Peptide name ^a	Peptide sequence ^b	AA ^c	Relative peptide affinity HSPA1A/HSPA8 ^d
K1	RKALDVITIAVP	12	++/++
K2	KSADTLWDIQDLKDL	16	++/++
K3	VRMILKIDDIRKPGSEEE	18	0/0
K4	ATETVRSILKIDDVVNTR	18	+/++
K5	TETVRSILKIDDVVNTR	17	++/++
K6	PAVLGFEGSANKIGVGVRD	20	+/++
K7	IIHVPQKED	9	+/++
K8-1	GGQSVYHVHLHVLGGRQMHV	20	0/0
K8-2	SVYHVHLHVLGGRQMHVPPG	20	0/0
K9-1	AGKHVPRAVFVDLEPTVIDE	20	+/++
K9-2	KHVPRAVFVDLEPTVIDEVR	20	+/++
K10	NSKIVVVTAGVRQEG	16	+/++
K11-1	DGRGGGPRSTAGGVALAVVV	20	++/++
K11-2	GGPRSTAGGVALAVVLSLA	20	++/++
K12	FHPEQLITGKEDAANNY	17	+/++
K13	GYTEHQVVSDFNSDTH	17	+/+
K14	VGKVIPELNGKLTGMA	16	+/++
K15	RVAPEEHPTLLTEAPLNPK	19	++/++
K16	VSQEEMEFIQRGGPE	15	0/+
K17	SVVPSPKVSDTVVEPYN	17	++/++
K18	KDILPRHIQ	9	++/++
K19	NILRASHSAVDIT	13	0/0
K20	VAPEEHPVLLTEAPLNPK	18	+/+
K21-1	EENINLFIKKVKNFLEENV	20	0/0
K21-2	ENINLFIKKVKNFLEENV	20	0/0
K22	VPLDPKLLLEDLVQP	14	++/++
K23	SEAFREANLAASFGKSIN	18	+/++
K24	ISKIREEYPDRIMNTF	16	0/0
K25	PPNPEEVKTPEEGEKQSQC	19	++/++
K26-1	YDPEAASAPGSGNPCHEASA	20	++/++
K26-2	ASAPGSGNPCHEASAAQKEN	20	++/++
K26-3	SGNPCHEASAAQKENAGEDP	20	++/++
K26-4	PCHEASAAQKENAGEDPGLA	20	++/++
C1-1	ISWYDNEFGYSNRVVDLMAH	20	0/0
C1-2	NEFGYSNRVVDLMAHMASKE	20	0/+
C2-1	YDNEFGYSNRVVDLMAHMAS	20	0/+
C2-2	NEFGYSNRVVDLMAHMASKE	20	0/+
C3-1	DNEFGYSNRVVDLMAHMASK	20	0/0
C3-2	NEFGYSNRVVDLMAHMASKE	20	0/+
C4	ISWYDNEFGYSNRVVDL	17	++/++
C5	DNEFGYSNRVVDLM	14	0/0
C6	DNEFGYSNRVVDL	13	0/+
C7	DAGAGIALNDHFVKL	15	++/++
C8	AGAGIALNDHFVKL	14	++/0
C9	GMAFRVPTANVSVDLT	17	+/++
C10	SEGPLKGILGY	11	++/++
C11	GILGYTEHQVVSDFNSDTH	20	++/++
C12	AVFVDLEPTVIDEVR	15	++/++
C13	WWVAYNDKP	9	++/++
C14	VSSTINPILYNL	12	+/++

Table 2 (continued)

Peptide name ^a	Peptide sequence ^b	AA ^c	Relative peptide affinity HSPA1A/HSPA8 ^d
C15	MKVEVLPALTDNY	13	0/+
C16	AGFAGDDAPRAVFPISVG	18	+/++
C17	MYEGLWMSVCSQSTG	15	+/++
C18	DKLKGEMMDL	10	0/+

^a Index name, K and C indicate the cell line from which the HSPA-associated peptide was eluted, K562 and CCRF-CEM, respectively. '-' peptides longer than 20 amino acids were truncated to 20 residue fragments with at least 15 amino acid overlapping fragments

^b Sequence of the peptide synthesised on the peptide array

^c Number of amino acids in the peptide synthesised on the peptide array

^d Relative binding affinity of recombinant HSPA1A or HSPA8 to the peptides; where 0 indicates none, + indicates moderate and ++ indicates high relative affinity. The peptides with either an intensity lower than the double average value of the negative control spots, or did not reach an intensity higher than a twofold increase over the corresponding control, were assessed as none binding (0). All the others were assessed as either moderate (+; when an intensity of higher than a twofold increase over the corresponding control was reached) or high affinity binders (++; when an intensity of higher than a fourfold increase over the corresponding control was reached; Fig. 4)

one amino acid mismatch to the reported epitopes, however not in an anchoring position (Table 4). All four (K15, K20, K24 and K26) of the HSPA-associated peptides which were matched to the reported HLA class I epitopes were isolated from the K562 cell line. From the HSPA-associated peptides, which were identified to be precursors for HLA class I epitopes, two were reported to be eluted from HLA-A*02 (K15-A and K20-A), three from HLA-B*35 (K15-B, K20-B and K24-B), two from HLA-B*3910 (K15-B and K20-B), one from HLA-A*2402 (K24-A) and one from HLA-B*27 (Table 4; Alvarez et al. 2007; Boisgerault et al. 1998; Kruger et al. 2005; Yague et al. 1998). K24-A and K24-B peptides were found to have a correct C-termini, whereas K15-A and K26-A had a correct N-termini with the reported class I epitopes. The HSPA-associated peptides which were matched with HLA class I epitopes were derived from actin α 1 precursor (K15-A and K15-B), actin β (K20-A and K20-B), tubulin β (K24-A and K24-B) and dermcidin (K26-A; Table 4). Four K562 HSPA-associated peptides may therefore be precursors for class I epitopes, indicating an involvement of HSPA in the HLA class I antigen presentation process.

The HSPA-associated peptides were searched against the databases of HLA class II reported epitopes. Two of the peptides (K15 and K20) were matched to HLA class II epitopes reported to be eluted from HLA-DR β *0404 (K15-C and K20-C) and HLA-DR β *0301 (K15-D and K20-D; Table 4; Kirschmann et al. 1995; Wahlstrom et al. 2007).

Table 3 Potential HLA class I epitopes restricted to the HLA type of CCRF-CEM cell line deduced from the sequences of the CCRF-CEM HSPA-associated peptides

Peptide name ^a	HLA Type ^b	Potential epitope ^c
C10	A*01	- <u>T</u> D - - - <u>L</u> - Y
		- <u>S</u> E - - - - -
	A*01	G P L K G I <u>L</u> G Y
	A*3101	- <u>L</u> <u>F</u> - - <u>L</u> - - R
		- <u>V</u> <u>L</u> - - <u>F</u> - - -
		- <u>Y</u> <u>Y</u> - - <u>V</u> - - -
C16	A*3101	G <u>F</u> A G D D A P R
	B*4001	- E - - - - <u>I</u> - L
		- - - - - <u>V</u> - -
C1-C6	B*4001	G Y S N R V <u>V</u> D L
C7, C8	B*4001	A L N D H F <u>V</u> K L
C9	B*4001	T A N V S V <u>V</u> D L
	C*0304	- A <u>V</u> <u>P</u> - - <u>M</u> - L
		- - <u>I</u> <u>E</u> - - <u>E</u> - M
		- - <u>P</u> <u>D</u> - - - - -
		- - <u>Y</u> - - - - -
		- - <u>A</u> - - - - -
C9	C*0304	T A N V S V V D L
C18	C*0304	K L K G E M <u>M</u> D L

K15-C and K20-C were missing only one amino acid at the C-terminal end, and K15-C had an additional amino acid mismatch (Table 4). K15-D and K20-D were missing two and three residues at the N-terminal end to the reported epitope, respectively. K15-D had an additional residual mismatch (Table 4). Peptide K20 was derived from actin β and K15 from actin $\alpha 1$ precursor. The minor sequence mismatch in terms of HLA class II was much less significant due to fewer restrictions in the peptide binding motif for HLA class II compared to HLA class I molecules and made these peptides strong candidates for HLA class II epitopes.

Discussion

This report provides the first analysis of a human repertoire of HSPA-associated peptides. We analysed the peptides isolated from purified cytosolic HSPA, containing the isoforms HSPA1A and HSPA8HSPA, for the presence of antigenic determinants. We provide evidence that HSPA captures peptide intermediates for HLA class I and class II molecules. Our results show a general characteristic of the HSPA chaperoned peptides and support the suggestion of involvement of HSPA in the antigen presentation process.

We expected HSPA binding specificity to be at least partially conserved, which was confirmed by the peptide repertoire of HSPA-associated peptides both between the cell lines and in comparison to the reported mouse HSPA-associated peptides (Grossmann et al. 2004). Two pairs of peptides, K9=C12 and K13=C11, isolated from both cell lines, were found to have overlapping sequences. In addition, peptides K15 and K20, although derived from two different proteins, showed a high level of sequence homology (Table 1). We also observed that HSPA binds preferentially to peptides derived from the C-terminal end of the proteins. Thirteen peptides described in our report were shown to share common sequences with peptides reported as co-purified with mouse HSPA (Grossmann et al. 2004; Table 1). These results show that in vivo selection of HSPA-associated peptides is not indiscriminate, which is in agreement with the report by Grossmann et al. (2004). There was no observed variation in the proportions of HSPA1A and HSPA8 between the cell lines; therefore, the different peptide repertoire was probably not related to this issue. The variations in the peptide repertoires, however, may be explained by the fact that these two cell lines are phenotypically diverse and represent two lineages of lymphoid and myeloid leukaemias. K562 has been identified as an erythromyeloblastoid

Table 4 Reported HLA epitopes deduced from the sequences of the HSPA-associated peptides

Peptide name ^a	HLA type ^b	Reported epitope ^c	Reference
Class I			
K15-A	A*02	RV AP V EEHPT/ V L	Kruger et al. (2005)
K15-B	B*35	A PEEHPT/ V LL	Alvarez et al. (2007)
K15-B	B*3910	A PEEHPT/ V LL	Yague et al. (1998)
K20-A	A*02	(R) V AP V EEHPVL	Kruger et al. (2005)
K20-B	B*35	A PEEHPVLL	Alvarez et al. (2007)
K20-B	B*3910	A PEEHPVLL	Yague et al. (1998)
K24-A	A*2402	E YPDRIMNTF	Alvarez et al. (2007)
K24-B	B*35	Y/ _F P DRIMNTF	Alvarez et al. (2007)
K26-A	B*27	Y/ _X D/ _X P EAAAPGSGNP/ _R	Boisgerault et al. (1998)
Class II			
K15-C	DRβ1*0404	V AP V EEHPT/ V LLTEAPLNPK(A)	Kirschmann et al. (1995)
K20-C	DRβ1*0404	V AP V EEHPVLLTEAPLNPK(A)	Kirschmann et al. (1995)
K15-D	DRβ1*0301	(EL) R VAP V EEHPT/ V LLT	Wahlstrom et al. (2007)
K20-D	DRβ1*0301	(ELR) V AP V EEHPVLLT	Wahlstrom et al. (2007)

^a Index name, K indicates K562 cell line from which the HSPA-associated peptide was eluted. ‘-’ truncated variant of the peptide

^b HLA type from which the reported HLA epitope was eluted

^c HLA epitopes sequences deduced from the HSPA-associated peptides. HLA epitopes were searched in SYFPEITHI and IEDB databases. **Bold**, residues matched to the reported HLA epitope and the HSPA associated peptide; (R), (A), (EL), (ELR)—residues were not present in the HSPA-associated peptide but were present in the matched HLA epitope; Y/_F, T/_V, P/_R—residual mismatch: subscript, bold residue was present in the reported HLA epitope, regular case amino acid was present in the HSPA associated peptide; Y/_X, D/_X—potential residual mismatch where X is a residue which was not identified

leukaemia cell line whereas CCRF-CEM as a T cell lymphoblast-like cell line; consequently, it can be speculated that these cell lines have different protein expression profiles and thus different peptide repertoires.

The peptide repertoire of HSPA depends on the protein expression profile of the cell and the HSPA specific binding affinity. Therefore, in order to gain further insight into the profile of the HSPA-associated peptides, we studied the preferential binding motif within the peptides. It has been reported that HSPA preferentially binds peptides with a hydrophobic core (Fourie et al. 1994; Grossmann et al. 2004; Maeda et al. 2007; Takenaka et al. 1995), with preference for basic (Fourie et al. 1994; Grossmann et al. 2004; Maeda et al. 2007; Takenaka et al. 1995) and large aromatic residues (Fourie et al. 1994), and that acidic amino acids are rarely present (Fourie et al. 1994; Maeda et al. 2007; Zhang et al. 2002). In our report, the HSPA-associated peptides revealed a hydrophobic arrangement, interspersed with hydrophilic residues, including acidic ones, and we did not find preferences for aromatic residues (Table 1). Therefore, our data, with reference to the HSPA binding motif, are in agreement with the in vivo study of Grossmann et al. (2004), but are in contrast to HSPA binding motifs which have been proposed in the in vitro studies (Fourie et al. 1994; Maeda et al. 2007; Takenaka et al. 1995; Zhang et al. 2002). The binding of the peptides co-purified with HSPA was

tested with recombinant HSPA1A and HSPA8 on a peptide array. The peptides presented a similar binding pattern between the two isoforms, HSPA1A and HSPA8 (Fig. 4 and Table 2). Although most of the peptides showed affinity to HSPA on the peptide array, a number of peptides were defined as non-binding. This potentially was caused by the lack of HSPA cofactors, such as DNAJ proteins, which are present in the cell and dictate the binding characteristic of HSPA (Cheetham and Caplan 1998; Johnson and Craig 2001; Sahi and Craig 2007). On the other hand, low-affinity binding peptides could be considered as contaminants; however, peptides C1, C2, C3, C5 and C6, which showed a low level of binding, contain common sequences with peptides which have been previously reported as co-purified with mouse HSPA (Grossmann et al. 2004; Table 1). The orientation of the peptides on the membrane, and the method of immobilising them, may have potentially reduced accessibility of the peptide binding site to the protein. What is more is that steric hindrance may have also reduced binding of HSPA to the membrane-attached peptides, thus limiting this assay.

The involvement of HSPs, including HSPA family members, in the antigen presentation process has been described and reviewed by Srivastava (2002). It has been shown that HSPA8 interacts directly with the TAP complex in an ATP-dependent manner, potentially feeding TAP with

chaperoned peptides (Kamiguchi et al. 2008). Although the premise that HSPA conveys chaperoned peptides directly to the TAP complex is appealing, further detailed investigations are needed. HSPA1A (Mycko et al. 2004) as well as HSPA8 (Panjwani et al. 1999; Zhou et al. 2005) have also been reported to enhance HLA class II antigen presentation and to localise in MIIC. Recently, different HSPs such as HSPC and CCT have been demonstrated to chaperone post-proteasomal antigenic intermediates for the HLA class I presentation pathway, indicating its role in the early steps of antigen presentation (Callahan et al. 2008; Kunisawa and Shastri 2003, 2006). It seems that particular HSPs have a common ability to shuttle post-proteasomal peptides, potentially protecting them from cytosolic peptidases and thus complete degradation. Peptides generated by the proteasome frequently have a correct C-terminus for HLA class I presentation and need to be trimmed only by aminopeptidases (Cascio et al. 2001). The K24 peptide, which was matched to two reported HLA class I epitopes, was found to have a correct C-terminus (Table 4). Three other peptides, K15, K20 and K26, matched to seven reported HLA class I epitopes would require generation of new C-termini (Table 4) potentially via the peptidase TPPII (Reits et al. 2004). These results suggest that HSPA might chaperone peptides from the proteasome to TPPII, if necessary, and to the TAP complex which would then convey the peptides into the ER. In a very similar manner, the HLA class II antigen presentation of endogenous epitopes might be handled by HSPA since it has been shown that endogenous class II epitopes are proteasome- and TAP-dependent (Mukherjee et al. 2001; Tewari et al. 2005). In fact, two peptides co-purified with HSPA, K15 and K20, were matched to HLA class II epitopes (Table 4), which consequently suggests that HLA class II epitopes may be chaperoned by HSPA. This also indicates that HLA class II epitopes can be generated by cells which do not express class II molecules. In respect to the HSPA-based anti-tumour therapy (Srivastava 2002), it suggests that HSPA delivers to APCs not only HLA class I but also HLA class II epitopes. Such APCs stimulated with HSPA-associated peptides would be able to induce not only CD8⁺ but also CD4⁺ T cell-mediated antigen-specific response. Accordingly, it is in agreement with the observation that depletion of CD4⁺ T cells abrogates the anti-tumour immune response elicited by HSPA with associated peptides (Sato et al. 2001; in that study, HSPA was purified using the ADP affinity method and thus contained both HSPA1A and HSPA8 family members; Peng et al. 1997).

In conclusion, we have shown that HSPA binds HLA class I and class II epitope precursors, which further strengthens the role of HSPA in the HLA class I and class II antigen presentation process. In addition, our findings are in agreement with the proposed role of HSPA as the HLA

class I and class II antigenic peptide carrier for cross-presentation (Sato et al. 2001; Srivastava 2002).

Acknowledgements We would like to thank to Dr. Joe Gray from the Pinnacle Lab, the Institute of Cell and Molecular Biosciences, Newcastle University, Newcastle upon Tyne, UK for the service and extensive help with the mass spectrometric analysis. This work was supported by grant from the Leukaemia Research Fund, UK [04070]; “Trans-Net” grant from the European Commission [MRTN-CT-2004-512253]; German Research Foundation (DFG) [MU1238/7-2] and the German Federal Ministry of Education and Research (BMBF) MOBITEM [01EZ0826] grants.

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