Biol. Chem., Vol. 390, pp. 305–312, April 2009 • Copyright © by Walter de Gruyter • Berlin • New York. DOI 10.1515/BC.2009.038

Interaction of human heat shock protein 70 with tumor-associated peptides

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Abstract

Molecular chaperones of the heat shock protein 70 (Hsp70) family play a crucial role in the presentation of exogenous antigenic peptides by antigen-presenting cells (APCs). In a combined biochemical and immunological approach, we characterize the biochemical interaction of tumor-associated peptides with human Hsp70 and show that the strength of this interaction determines the efficacy of immunological cross-presentation of the antigenic sequences by APCs. A fluorescein-labeled cytosolic mammalian Hsc70 binding peptide is shown to interact with human Hsp70 molecules with high affinity (K_d=0.58 μM at 25°C). Competition experiments demonstrate weaker binding by Hsp70 of antigenic peptides derived from the tumor-associated proteins tyrosinase (K_d=32 μM) and melanoma antigen recognized by T cells (MART-1) (K_d=2.4 μM). Adding a peptide sequence (pep70) with high Hsp70 binding affinity (K_d=0.04 μM) to the tumor-associated peptides enables them to strongly interact with Hsp70. Presentation of tumor-associated peptides by B cells resulting in T cell activation *in vitro* is enhanced by Hsp70 when the tumor-associated peptides contain the Hsp70 binding sequence. This observation has relevance for vaccine design, as augmented transfer of tumor-associated antigens to APCs is closely linked to the vaccine's efficacy of T cell stimulation.

Keywords: antigen presentation; antigenic peptide; anti-tumor T cell response; heat shock protein 70; molecular chaperone; peptide binding.

Introduction

The highly conserved 70 kDa heat shock proteins (Hsp70) are molecular chaperones involved in the folding of newly synthesized polypeptides, refolding of misfolded proteins, membrane translocation and control of regulatory proteins (Hartl and Hayer-Hartl, 2002; Mayer and Bukau, 2005). They achieve their functions through interaction with short hydrophobic stretches within protein substrates, and are capable of binding short peptides *in vitro* (Flynn et al., 1991; Landry et al., 1992; Blond-Elguindi et al., 1993; Gragerov and Gottesman, 1994; Rüdiger et al., 1997; Knarr et al., 1999). Both stress-inducible Hsp70 and constitutively expressed 70 kDa heat shock cognate (Hsc70) isoforms occur in the cytoplasm of mammalian cells.

Hsp70 molecules possess a highly conserved amino (N)-terminal ATPase domain which regulates the opening and closing of the carboxy (C)-terminal peptide binding domain. It appears that the adenosine triphosphate (ATP)-bound form of Hsp70 has low affinity and fast exchange rates for substrate proteins and peptides, and undergoes slow hydrolysis to the adenosine diphosphate (ADP)-bound form, which has high affinity and low exchange rates for substrates (Mayer and Bukau, 2005). The substrate-binding domain consists of an 8-stranded antiparallel β-sandwich with a hydrophobic cavity, covered by an α-helical 'lid' domain (Zhu et al., 1996; Stevens et al., 2003).

The crystal and solution structures of complexes of the substrate-binding domain with a bound peptide suggest that backbone hydrogen bonding holds an extended pentapeptide in the cavity of bacterial Hsp70 (DnaK), with a central leucine residue accommodated in a deep hydrophobic pocket (Zhu et al., 1996; Stevens et al., 2003). Presumably ATP binding triggers opening of the binding cavity, facilitating substrate release.

Recent work suggests localization of Hsp70 to the plasma membrane of cancer cells and a role for extracellular Hsp70 in anti-tumor immunity (Milani et al., 2002; Multhoff, 2002). Molecular chaperones isolated from tumor cells can elicit anti-tumor immunity *in vivo* and *in vitro* (Udono and Srivastava, 1993, 1994; Suto and Srivastava, 1995). They were found to be associated with antigenic peptides (Ishii et al., 1999), which they appear to transport to antigen-presenting cells (APCs) for cross-presentation (Castellino et al., 2000; Noessner et al.,

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2002; Ueda et al., 2004; Zheng and Li, 2004). The presentation of exogenous antigens by major histocompatibility complex (MHC) class I molecules on the APC surface leads to specific anti-tumor immunity (Calderwood et al., 2005). Antigenic peptides vary in their hydrophobic content and their binding affinity for molecular chaperones. Enhanced delivery of exogenous antigens by Hsp70 to APCs leads to stronger T cell responses (Bendz et al., 2007). This function can be utilized in the generation of protein-based vaccines that are intended to stimulate antigen-specific CD8 T cell responses. Fusion of the antigenic peptides with a motif known to bind BiP (Flynn et al., 1991), an Hsp70 isoform in the endoplasmic reticulum (also named grp78), has been used to enhance their interaction with Hsp70 (Moroi et al., 2000; Flechtner et al., 2006). Enhancement of antigen-specific T cell stimulation was shown to depend on the degree of complex formation between Hsp70 and peptides (Bendz et al., 2007). As the physiological capacity of APCs to cross-present antigen is generally low (Maecker et al., 2001), there is significant interest in reagents and mechanisms that enhance the targeting of exogenous antigens to APCs. An improved understanding of the interaction of Hsp70 molecules with antigenic peptides will therefore aid the development of more effective vaccines.

We developed an assay to analyze the interaction of human Hsp70 with tumor-associated peptides by comparison with a standard peptide known to bind cytosolic mammalian Hsc70 and human Hsp70 (Fourie et al., 1994; Takeda and McKay, 1996; Davis et al., 1999). Since extracellular ATP levels are generally low, our study focuses on the nucleotide-free state of Hsp70, which is known to have high affinity for substrate. The equilibrium binding parameters determined show that the affinity of the antigenic peptide molecules that bind to the chaperone protein can be related to the efficacy of being presented by human APCs for T cell stimulation in the context of Hsp70 as delivery vehicle.

Results and discussion

Peptide binding by human Hsp70

Recombinantly expressed human Hsp70 was purified and shown to possess ATPase activity (data not shown) consistent with the literature (Wegele et al., 2003). Absorbance spectroscopy of the purified recombinant human Hsp70 shows a spectrum typical of a protein sample with no obvious contribution of nucleotides to the signal at 260 nm (data not shown). In order to analyze peptide binding to Hsp70, we synthesized a peptide (AF-1, see Table 1) with the amino acid sequence FYQLALT (single letter code), which has been used previously to study cytosolic mammalian Hsc70 binding (Takeda and McKay, 1996). Size exclusion chromatography has previously been used to quantify binding of AF-1 peptide labeled with fluorescein at the N-terminus (Takeda and McKay, 1996). Using size exclusion chromatography, we found that human Hsp70 was a mixture of monomer and dimer typical for this protein (data not shown). Fluorescein-labeled AF-1 (FAF-1) was detected by fluorescence signal and seen to elute much later than Hsp70, at a much higher volume than the total column volume. It has been suggested that the elution volume of FAF-1 was greater than the column volume in the presence of 200 mM KCl due to salt-dependent hydrophobic interaction of the peptide with the column matrix (Takeda and McKay, 1996). Incubation of Hsp70 with FAF-1 clearly leads to peptide binding, as a fluorescence signal is detected at the retention time of free Hsp70. In the presence of excess unlabelled peptide (AF-1), a diminished fluorescence signal is observed at the retention time of free Hsp70. The competition of unlabelled peptide for Hsp70 demonstrates specific binding of the peptide; however, quantitative data were not derived from this assay. Therefore, we developed a spectroscopic assay to compare the Hsp70 binding affinities of synthetic peptides at equilibrium.

Quenching of the fluorescence emission of the labeled FAF-1 peptide is observed upon interaction with Hsp70, with up to 20% loss in signal (data not shown). Titration of the protein into a peptide solution results in a concentration-dependent decrease in fluorescence signal which can be quantified. Equilibrium binding parameters for FAF-1 interaction with human Hsp70 in the nucleotide-free state were calculated from fluorescence titrations by fitting the data to binding equations (Table 1). While a dissociation constant (K_d) of 5.0 ($\pm$0.9) μM was determined at 37°C, binding is clearly stronger at 25°C with a K_d of 0.58 ($\pm$0.10) μM (Figure 1). The value at 25°C was confirmed by performing a titration with FAF-1 at five times lower concentration, yielding a K_d of 0.36 ($\pm$0.08) μM (Figure 2). A comparable increase in equilibrium con-

Table 1 Equilibrium binding parameters of peptide interaction with human Hsp70.

Additive	Peptide	Sequence[a]	K_d (μM)	
			25°C	37°C
	FAF-1	f-FYQLALT	0.58±0.10	5.0±0.9
1 mM ADP	FAF-1	f-FYQLALT	1.7±0.2	
	AF-1	FYQLALT	0.87±0.06	2.3±0.1
	pep70	HWDFAWPW	0.039±0.006	
	Tyrosinase	YMNGTMSQV	31.6±4.5	
	MART-L	LAGIGILTV	1.6±0.2	
	MART-1	AAGIGILTV	2.4±0.8	
	pep70-tyrosinase	b-GSGHWDFAWPWGSGYMNGTMSQV	0.056±0.007	
	pep70-MART-L	b-GSGHWDFAWPWGSGLAGIGILTV	0.093±0.022	

[a]f, fluorescein; b, biotin.

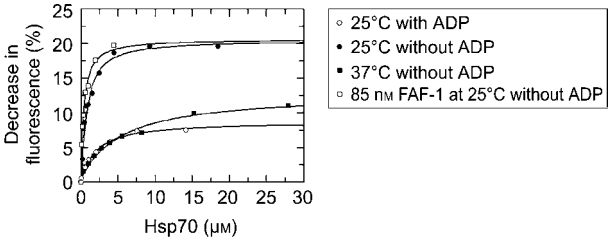

Figure 1 Interaction of human Hsp70 with a cytosolic mammalian Hsc70 binding peptide.
Fluorescence titrations of 0.5 μM FAF-1 peptide with Hsp70 at 25°C in the presence of 1 mM ADP and at 25°C or 37°C in the absence of ADP. A titration with 0.085 μM FAF-1 was performed at 25°C.

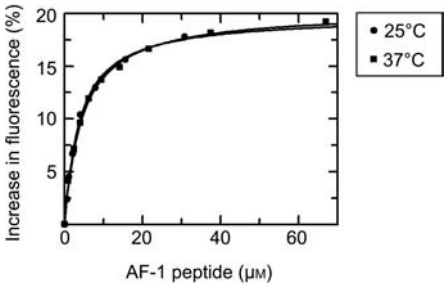

Figure 2 Competition of unlabeled peptide with a cytosolic mammalian Hsc70 binding peptide.
Fluorescence titrations of unlabeled competitor peptide (AF-1) with 0.25 μM and 2 μM Hsp70 at 25°C, or with 0.5 μM FAF-1 and 5 μM Hsp70 at 37°C.

stant at higher temperature has been reported for a DnaK-peptide complex (Farr et al., 1995). The large activation enthalpies observed for peptide association and dissociation (Farr et al., 1995) are consistent with the major conformational changes associated with substrate exchange (Stevens et al., 2003).

ADP-bound and nucleotide-free states of Hsp70 are known to have high affinity for substrate (Mayer and Bukau, 2005). To quantify the effects of the nucleotide state upon peptide binding, we performed fluorescence titrations in the presence of ADP or ATP. The initial fluorescence emission of FAF-1 peptide before titration with protein is reduced in the presence of nucleotide (data not shown). Binding is weaker in the presence of ADP (use of an ATP-depleting system as described in the materials and methods section removed any contaminating ATP) with a K_d of 1.7 (±0.2) μM at 25°C (Figure 1), and much weaker in the presence of ATP. The rapid exponential increase in fluorescence signal observed upon addition of ATP to a mixture of FAF-1 and Hsp70 is attributed to peptide release, demonstrating that the assay system responds in a reversible manner (data not shown). Previously, the FAF-1 peptide was shown to bind mammalian Hsc70 with a K_d of 4.3 μM in the presence of ADP (Takeda and McKay, 1996). Hsp70 and Hsc70 isoforms are conserved in the residues believed to be responsible for peptide binding specificity and allosteric communication (Mayer et al., 2000; Fernández-Sáiz et al., 2006), so their binding affinities are not expected to differ greatly. The lower K_d value determined here may reflect the use of an equilibrium technique different to the binding assays employed in previous work.

To compare the binding affinities of different peptides, unlabelled peptide was added to FAF-1 complexed with Hsp70 under simple binding conditions. Competition for Hsp70 binding leads to release of FAF-1 molecules and a resultant fluorescence increase. Fluorescence titration of unlabeled competitor peptide demonstrates that binding is reversible and specific. Using this novel assay, we found that AF-1 peptide bound Hsp70 with K_d values of 2.3 (±0.1) μM at 37°C and 0.87 (±0.06) μM at 25°C (Figure 2). The binding parameters obtained for AF-1 peptide from competition experiments clearly agree with the FAF-1 binding data (Table 1).

Binding of antigenic peptides

Antigenic peptides derived from tyrosinase protein and melanoma antigen recognized by T cells (MART-1), with the amino acid sequences YMNGTMSQV and AAGIGILTV, are recognized on human leukocyte antigen (HLA)-A2 positive melanomas by cytotoxic T lymphocytes (Kawakami et al., 1994; Wölfel et al., 1994). Although tumor-associated peptides were shown to be associated with human Hsp70 (Ishii et al., 1999), their interaction has not been reliably quantified. To compare the affinity of the tyrosinase and MART-1 epitopes for Hsp70, we performed competition experiments with synthetic peptides. Fluorescence titration of unlabeled competitor peptide with FAF-1 and human Hsp70 at 25°C yielded a K_d value of 31.6 (±4.5) μM (Figure 3), for tyrosinase peptide. Clearly, the Hsp70 binding affinity of the tumor-associated peptide is weaker than the standard peptide. Antigen presentation studies show that modification of the MART-1 epitope sequence to LAGIGILTV (MART-L) improves binding by HLA-A2 class I histocompatibility molecules (Rivoltini et al., 1999). To determine whether this amino acid substitution affects binding of the peptide to Hsp70, we performed a fluorescence titration (Figure 3). Hsp70 binding of the modified and natural epitopes was found to be similar with a K_d of 1.6 (±0.2) μM determined for the modified MART-L peptide and 2.4 (±0.8) μM for the natural MART-1 peptide (Figure 3).

However, the antigenic peptides derived from the tumor-associated proteins tyrosinase and MART-1 clearly differ in their Hsp70 binding affinities (Table 1). This dif-

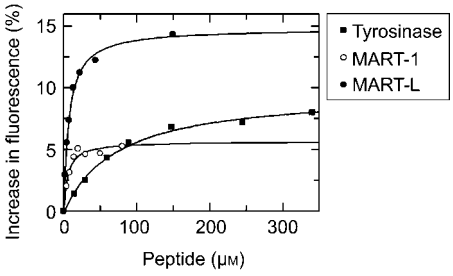

Figure 3 Interaction of human Hsp70 with antigenic peptides from tyrosinase protein and melanoma antigen recognized by T cells at 25°C.
Fluorescence titrations of unlabeled tyrosinase and MART-1 peptides with 0.2 μM FAF-1 and 0.75 μM Hsp70, and of unlabeled MART-L peptide with 0.25 μM FAF-1 and 2 μM Hsp70.

ference reflects their increasing hydrophobic content to some extent.

Binding of hybrid peptides

High transfer of antigen to APCs for MHC class I presentation is desirable for vaccine development. Hybrid peptides containing the antigenic sequence of the T cell epitope and a BiP binding motif (here named pep70, amino acid sequence HWDFAWPW) (Flynn et al., 1991) have been used to enhance their interaction with Hsp70 (Moroi et al., 2000; Flechtner et al., 2006). In our assay, tight binding was observed for the pep70 peptide alone, with a K_d value of 0.039 (±0.006) μM at 25°C (Figure 4). Obviously, the pep70 sequence binds to Hsp70 much more tightly than any of the investigated antigenic peptide sequences (Table 1). Previously, dissociation constants of 0.04 to 0.2 μM were measured between DnaK and peptides derived from region C of the heat shock transcription factor σ^{32} in a competition assay (McCarty et al., 1996).

The determined K_d values of the peptide sequences for binding to Hsp70 can be related to their amino acid sequence. BiP-binding peptides were shown to be rich in hydrophobic amino acids, particularly leucine or tryptophan (Knarr et al., 1999), and a motif has been proposed with the sequence Hy-(W/*X*)-Hy-*X*-Hy-*X*-Hy, where Hy is a large hydrophobic or aromatic amino acid and *X* is any amino acid (Fourie et al., 1994). Studies with DnaK identify a motif with a core of four or five hydrophobic residues, where leucine is particularly enriched, with flanking regions enriched in basic residues (Rüdiger et al., 1997). Although it is clear that tyrosinase peptide lacks core hydrophobic residues, the MART-1 and pep70 peptides have a similar content of hydrophobic side chains. However, MART-1 peptide contains just one preferred leucine and AF-1 contains two leucines, compared to three tryptophans in the pep70 sequence. In addition, the pep70 sequence contains large aromatic side chains at alternate positions which may account for its 60-fold stronger binding with Hsp70 than shown by the epitope.

Hybrid peptides combining the pep70 sequence with the natural tyrosinase (pep70-tyrosinase) and modified MART-1 epitopes (pep70-MART-L) were produced and their interaction with Hsp70 was determined. Using fluorescence titrations (Figure 4), we determined K_d values of 0.056 (±0.007) μM for pep70-tyrosinase and 0.093 (±0.022) μM for pep70-MART-L, which are much lower than for the individual nonameric T cell epitope sequences (Table 1), confirming that the pep70 sequence confers a strong interaction with Hsp70. Although the residues in the hybrid peptide that bind Hsp70 are not known, it is most likely that the binding observed occurs through the pep70 motif, particularly for the pep70-tyrosinase peptide, as the nonameric tyrosinase sequence is largely devoid of Hsp70 interacting amino acids.

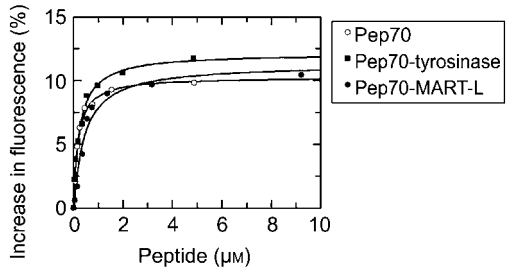

Figure 4 Interaction of human Hsp70 with a Hsp70 binding peptide (pep70) and pep70-hybrid peptides.
Fluorescence titrations of unlabeled pep70, pep70-tyrosinase and pep70-MART-L peptides with 0.25 μM FAF-1 and 2 μM Hsp70 at 25°C.

Antigen cross-presentation by B-lymphoblastoid cells in the presence of Hsp70

Next, we determined the effect of peptide:Hsp70 binding affinity on the efficacy of peptide cross-presentation in the context of Hsp70 as delivery vehicle (Figure 5). We used our previously established cross-presentation system which includes B-lymphoblastoid cell lines (B-LCL) L724.45 or L721.174 as APCs exposed to the same amount of peptide either as peptide:Hsp70 complexes or as peptide alone (Bendz et al., 2007). The Hsp70-mediated enhancement of antigen cross-presentation was compared between peptides, for which different binding efficacies to Hsp70 had been demonstrated (Table 1). The efficacy of antigen cross-presentation was determined by measuring the gamma interferon (IFN-γ) secretion of antigen-specific T cells. Antigen-specific T cells secrete IFN-γ after exposure to APCs and the amount of IFN-γ release correlates to the amount of presented antigen. To define whether the affinity by which the peptide binds to Hsp70 contributes to the cross-presentation efficacy, IFN-γ secretion obtained with peptide:Hsp70 complex-loaded APC was normalized to that obtained with peptide in the absence of Hsp70.

As seen in Figure 5 for the tyrosinase-specific T cell, a greater than two-fold enhancement in IFN-γ release was observed for the pep70-tyrosinase hybrid peptide when pre-incubated with Hsp70 to allow peptide:Hsp70 complex formation, while Hsp70 achieved no enhancement for the tyrosinase epitope which lacked the Hsp70 binding sequence. Similar results were obtained when com-

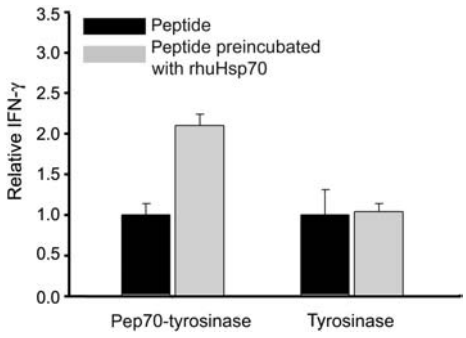

Figure 5 Hsp70-binding enhances cross-presentation of tyrosinase peptide.
B-LCL L724.45 cells were incubated with tyrosinase or pep70-tyrosinase peptide alone (dark bars) or pre-incubated with Hsp70 to allow complex formation (light bars). IFN-γ secretion by the antigen-specific T cell clone TyrF8 was measured by ELISA. The relative IFN-γ release is presented which is derived through normalization to the IFN-γ release achieved with the peptides alone; bars represent the mean of triplicate determinations±mean deviation.

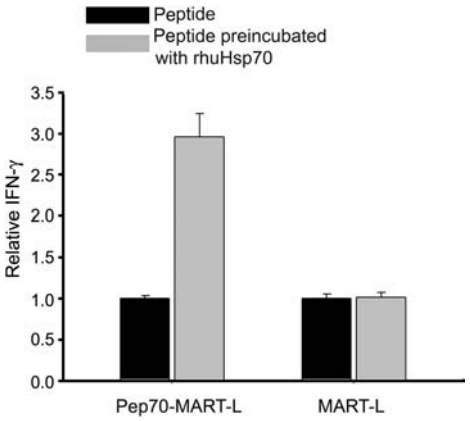

Figure 6 Hsp70-binding enhances cross-presentation of MART-L peptide.
B-LCL L724.174 cells were incubated with MART-L or pep70-MART-L peptide alone (dark bars) or pre-incubated with Hsp70 to allow complex formation (light bars). IFN-γ secretion by the antigen-specific A42-CTL was measured by ELISA. The relative IFN-γ release is presented which is derived through normalization to the IFN-γ release achieved with the peptides alone; bars represent the mean of triplicates±mean deviation.

paring the Hsp70-assisted cross-presentation of pep70-MART-L with MART-L (Figure 6). Thus, Hsp70 only enhanced cross-presentation of pep70-hybrid peptides containing the Hsp70-binding sequence, while no effect of Hsp70 was seen for peptide nonamers which lacked this sequence and have very low binding to Hsp70. The pep70 sequence itself does not interfere with the T cell stimulation (not shown), because it neither has an appropriate amino acid motif to bind to the HLA-A2 molecule nor does it have a sequence that stimulates the tyrosinase or MART-1-specific T cell receptors.

The binding parameters determined for the individual peptide sequences (Table 1) correlate with the capacity of Hsp70 to enhance cross-presentation of exogenous peptide (Figures 5 and 6). Although the effects of increasing temperature and volume following addition of the APCs need to be considered to fully understand the relationship between peptide binding affinity and antigen presentation, temperature- and volume-associated effects were minimized in our system, as peptide:Hsp70 complexes were always pre-formed in identical volume, buffer and temperature (25°C) before exposure to APC for uptake and antigen presentation. In our previous study, we analyzed the mechanism of Hsp70-assisted cross-presentation in some detail. We were able to show that Hsp70-mediated enhancement was dependent on peptide:Hsp70 complex formation resulting in enhanced uptake into the APC (Bendz et al., 2007).

Our results document that in an *in vitro* setting of vaccine generation, the antigen delivery to the APC can be optimized by using designed hybrid peptides together with Hsp70 as delivery vehicle. Multivalency of the T cell response is the desirable outcome of vaccination settings. This requires APC to present a multitude of different epitopes. Due to different uptake properties, peptides of a mixture are generally not presented equally resulting in T cell bias. Endowing peptides with an identical Hsp70 binding sequence and using Hsp70 as delivery vehicle will aid equal uptake of the peptides within a mixture which is a desirable property for vaccine design.

In summary, we describe a useful spectroscopic assay for measuring dissociation constants less than 100 μM between peptide and Hsp70. Using this new assay, we show that tumor-associated peptides differ in their binding affinities for human Hsp70. Fusion of the antigenic epitope with a Hsp70-binding motif confers a strong interaction with Hsp70 to give dissociation constants of 60–90 nM. Peptides with stronger binding to Hsp70 are better cross-presented and lead to stronger T cell activation than peptides which lack this sequence and have lower binding to Hsp70. Antigen cross-presentation is the required pathway for the generation of protein-based vaccines that are intended to stimulate antigen-specific CD8 T cells. One critical parameter, which defines the potency of T cell stimulation, is the efficiency of delivery of exogenous antigen to the APC. Our observation suggests that vaccine efficacy can be improved by using antigenic sequences containing the Hsp70 binding motif in the context of Hsp70 as an antigen delivery vehicle.

Materials and methods

Purification of Hsp70

DnaK-deficient *Escherichia coli* strain WKG191 (Kelley and Georgopoulos, 1997) cells transformed with plasmid pMPM-A4 (Mayer, 1995) expressing human Hsp70 were grown at 30°C in Luria-Bertani medium containing ampicillin (100 μg/ml) and induced overnight with 0.1% (w/v) L-arabinose. Cells were disrupted in 50 mM Tris-HCl, pH 7.6, containing 5 mM dithiothreitol and protease inhibitor mix G (Serva, Heidelberg, Germany) and the extract was centrifuged (40 000 *g*, 40 min at 4°C). The supernatant was separated by anion exchange chromatography at 4°C on a column (2.5×10 cm) of Q Sepharose Fast Flow (GE Healthcare Life Sciences, Munich, Germany) equilibrated in 25 mM HEPES/KOH, pH 7.6, 50 mM KCl, 5 mM $MgCl_2$, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM 2-mercaptoethanol. After washing the column with starting buffer, a linear gradient of 50–500 mM KCl over 500 ml was applied. The Hsp70-enriched fraction was identified by Tris/glycine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in the presence of the reducing agent, 2-mercaptoethanol (Laemmli, 1970). Hsp70 was precipitated by slowly adding 100% ammonium sulfate solution at 4°C with stirring to yield a final concentration of 60% and centrifuged (40 000 *g*, 40 min at 4°C). The pellet was resuspended in 10 mM K phosphate buffer, pH 7, dialyzed (Spectra/Por membrane, molecular weight cut-off 6–8000) against the same buffer, and applied to a column (1.5×14 cm) of hydroxyapatite (Bio-Rad, Munich, Germany) equilibrated in the same buffer at 4°C. After washing the column with starting buffer, a linear gradient of 10–400 mM K phosphate buffer, pH 7, over 500 ml was applied. After SDS-PAGE analysis, purified Hsp70 was dialyzed against 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$ at 4°C, then concentrated with an Amicon stirred cell (Ultracel YM-30 ultrafiltration membrane; Millipore, Schwalbach, Germany). Protein stocks were stored at -80°C and concentrations were estimated by absorption at 280 nm.

The identity and integrity of the purified Hsp70 was confirmed by matrix assisted laser desorption ionization mass spectrometry, circular dichroism spectroscopy and Western analysis using mouse anti-human Hsp70 monoclonal antibody (Affinity

BioReagents, Thermo Fisher Scientific, Rockford, USA) and its activity was demonstrated using a spectrophotometric ATPase assay (Norby, 1988).

Peptide synthesis

Peptides were synthesized using solid-phase methods and Fmoc chemistry. AF-1 peptide was labeled with fluorescein at the N-terminus by stirring a solution of unprotected peptide (0.053 mmol) in 1 ml *N*,*N*-dimethylformamide, adding 1.5 equivalents *N*,*N*-diisopropylethylamine followed by 1.5 equivalents fluorescein isothiocyanate (Sigma, Munich, Germany), incubating overnight at room temperature and then removing solvent in a vacuum.

Peptides were purified using reversed-phase high performance liquid chromatography, and their identities were confirmed by electrospray ionization mass spectrometry. Lyophilized peptide was dissolved in 4 M guanidinium chloride, pH 7, to prevent fibril formation and concentrated stock solutions were stored at -20°C. Sample concentrations were determined from their absorbance at 280 nm for unlabeled peptide or at 493 nm (ε=7.5×10^4 M^{-1} cm^{-1}; Carilli et al., 1982; Bernhardt et al., 1983) for fluorescein-labeled AF-1 (FAF-1), or from weight when no chromophore was present (MART peptides).

Biotin-pep70-tyrosinase and b-pep70-MART-L peptides were supplied by Biosyntan GmbH (Berlin, Germany) or the University of Munich Gene Center (Munich, Germany).

Size exclusion chromatography of peptide and Hsp70

Samples of 5 μM FAF-1 alone, 10 μM Hsp70 alone, and mixtures of 5 μM FAF-1 and 10 μM Hsp70 in the presence and absence of 25 μM AF-1 were incubated for 90 min at 25°C. High performance size exclusion chromatography of the samples was performed using a Superdex 200 HR 10/30 column, calibrated with a total volume of 24 ml, at a flow rate of 0.75 ml min^{-1} in 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$ at 25°C, with detection of protein absorbance at 280 nm and fluorescein fluorescence (excitation, 492 nm; emission, 512 nm). The column was calibrated by running standards (dextran, bovine serum albumin, ferritin, chymotrypsinogen) under the same conditions.

Fluorescence titration of peptide with Hsp70

Fluorescence emission data were recorded with a FluoroMax-2 fluorescence spectrometer (excitation, 492 nm; emission, 512 nm). Samples of FAF-1 at 85–500 nM (see legend to Figure 1) in 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, were analyzed in a stirred cuvette at 25°C or 37°C. Initial experiments in the presence of commercial ADP suggested the presence of contaminating ATP; therefore, we used an ATP-depleting system (hexokinase/glucose) for our titrations. A 1.5-ml sample containing 0.5 μM FAF-1 peptide in the presence of 1 mM ADP, 10 mM glucose and 7.5 U hexokinase was titrated with Hsp70. Data were collected after incubation periods of 15–210 min following addition of aliquots of Hsp70 (at concentrations of 125 or 350 μM), depending on the relaxation kinetics of the system being measured, and signal over a period of 200–1000 s was averaged. Experiments were repeated and control samples of FAF-1 incubated under identical conditions did not show loss of fluorescence signal with time. Fluorescence signal, corrected for dilution during titration, was expressed as a percentage fluorescence change and plotted against the total concentration of Hsp70. Data were fitted to a quadratic binding equation for fluorescence quenching using the Marquardt nonlinear least-squares algorithm within the GraFit 3.00 analysis software (Erithacus Software Ltd., East Grinstead, UK):

$$F=F_a\cdot\alpha+F_0,$$

where F_0 is the fluorescence intensity and F_a is the amplitude of the change in signal upon binding, and α is the fraction of FAF-1 peptide molecules in the bound state:

$$\alpha=\frac{P_0+L_0+K_d-\sqrt{(P_0+L_0+K_d)^2-4P_0\cdot L_0}}{2P_0},$$

where P_0 is the total concentration of FAF-1 peptide; L_0 is the total concentration of Hsp70; and K_d is the dissociation constant.

Fluorescence titration of unlabeled competitor peptide

Samples of FAF-1 at a concentration (200–500 nM, detailed in legends of Figures 2–4) below the K_d (simple binding conditions) were incubated with Hsp70 (750 nM–5 μM, detailed in the legends of Figures 2–4) in 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$, in a stirred cuvette at 25°C or 37°C. Fluorescence emission data were collected as described after incubation periods of 25–180 min following addition of aliquots of unlabelled peptide at the following concentrations (or dilutions thereof): 2 mM AF-1; 3.4 mM pep70; 16 mM tyrosinase; 11.6 mM MART-L; 15.5 mM MART-1; 1.2 mM pep70-GSG; 5.4 mM pep70-tyrosinase; 6.3 mM pep70-MART-L. Experiments were repeated. Fluorescence signal corrected for dilution was expressed as a percentage fluorescence change, plotted against the total concentration of competitor peptide (*I*) and fitted with fixed K_d to the equation:

$$F=F_0+F_a\cdot\left(\frac{L_0}{L_0+K_d\cdot\left(1+\frac{I}{K_i}\right)}\right)$$

where K_i is the dissociation constant for the *I:L* complex.

In vitro cross-presentation assay

Cross-presentation was performed as described recently using highly purified recombinant human Hsp70 (Bendz et al., 2007) and peptides and APCs as indicated. In brief, 10 μM pep70-tyrosinase peptide or 0.05 μM tyrosinase peptide, or 70 nM pep70-MART-L peptide or 2 μM MART-L in 25 mM HEPES/KOH, pH 7.6, 150 mM KCl, 5 mM $MgCl_2$ was added to wells of a 96-well cell culture plate and pre-incubated with 1 μM Hsp70 or a corresponding volume of buffer at 25°C. After 4 h, B-LCL L724.45 or L721.174 cells (15 000 in 90 μl AIM-V medium) were added, and incubated at 37°C to allow uptake. After 1 h, the HLA-A*0201-restricted tyrosinase peptide Tyr$^{368\text{-}376}$ (YMNGTMSQV)-specific cytotoxic T cell clone TyrF8 (kindly provided by Dr. P. Schrier, Department of Clinical Oncology, Leiden University Hospital, Leiden, Netherlands) (Visseren et al., 1995) or Melan-A/MART-1 peptide$^{27\text{-}35}$ (AAGIGILTV)-specific A42-CTL (kindly provided by M.C. Panelli, National Institute of Health, Bethesda, MD, USA) were added (4000 cells/90 μl) in RPMI medium containing 10% fetal calf serum, 10% human serum and rIL-2 (50 U/ml; Proleukine, Cetus Corp., Emeryville, CA, USA). After 24 h at 37°C, supernatants were harvested and the content of IFN-γ was measured by ELISA (OptEIA™; Pharmingen, San Diego, USA). Control samples, containing all components except the peptide, were used to determine IFN-γ background, which was subtracted from the experimental sample.

Acknowledgments

We thank Stefanie Hufnagl for assistance with protein purification, Helmut Krause for performing mass spectrometry, Daniel

Neumaier and Anna Brandl for the T and B cell cultures. The clone for human Hsp70 was kindly provided by Matthias Mayer, Molecular Biology Center, University of Heidelberg, Germany. We are grateful to Stefan Walter for helpful discussions. This work was supported by the Deutsche Forschungsgemeinschaft SFB455 (to E.N. and R.D.I.).

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Received October 28, 2008; accepted January 19, 2009