Dissertation zur Erlangung des Doktorgrades

der Fakultät für Chemie und Pharmazie

der Ludwig-Maximilians-Universität München

Mechanisms of Hsp70-Mediated Antigen Cross-Presentation

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aus

Tel Aviv

2007

Erklärung

Diese Dissertation wurde im Sinne von § 13 Abs. 3 bzw. 4 der Promotionsordnung vom 29. Januar 1998 von Herrn Prof. F. Ulrich Hartl betreut.

Ehrenwörtliche Versicherung

Diese Dissertation wurde selbständig, ohne unerlaubte Hilfe erarbeitet.

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Dissertation eingereicht am	15.03.2007
1. Gutachter:	Prof. Dr. F. Ulrich Hartl
2. Gutachter	PD Dr. Konstanze Winklhofer
Mündliche Prüfung am	08.05.2007

DANKSAGUNG

Die vorliegende Arbeit wurde in der Zeit von November 2002 bis Dezember 2006 am Max-Planck-Institut für Biochemie, Martinsried, in der Abteilung Zelluläre Biochemie angefertigt.

Mein besonderer Dank gilt Herrn Prof. Dr. F. Ulrich Hartl für die Betreuung meiner Promotion, für die Bereitstellung des interessanten Themas und der ausgezeichneten Arbeitsbedingungen, für die zahlreichen Diskussionen sowie für seine stete Unterstützung. Für hilfreiche Ratschläge und das freundliche Arbeitsklima danke ich auch Dr. Manajit Hayer-Hartl.

Besonders herzlicher Dank gilt Dr. Konstanze F. Winklhofer für ihre Unterstützung während meiner Promotion und für die Erstellung des Zweitgutachtens.

Andrea Obermayr-Rauter, Silke Leutz-Bütün und Bernd Grampp danke ich dafür, dass sie das Labor am Laufen halten.

Besonderer Dank gilt ebenfalls Prof. Dr. Felix Wieland, Sandra Zitzler und Dr. Petra Distelkötter-Bachert für die erfolgreiche Zusammenarbeit an diesem Projekt.

Für viele glückliche Stunden in- und außerhalb des Labors möchte ich mich bei allen momentanen und ehemaligen Kollegen bedanken, insbesondere danke ich in diesem Zusammenhang Angela, Chris, Christian, Dirk, Geli, Gregor, Jason, Jose, Juliane, Iris, Katja, Kausik, Luis, Michi, Nagore, Nikolay, Pen, Peter, Satish, Shruti, Sophia, Tobi, Ulrike und Vishwas.

Von ganzem Herzen danke ich auch meiner Familie, die mir immer eine große Unterstützung war. Mein größter Dank gilt meinem Mann Roderich Hellwig, der mir zu allen Zeiten zur Seite stand und mein Leben mit Glück und Liebe erfüllt.

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I SUMMARY

Hsp70 is a highly conserved, ubiquitous molecule and its properties as a molecular chaperone are well-described. Additionally, it has been shown to carry out important functions in the mammalian immune system. This study addressed several open questions concerning the interaction of Hsp70 with the surface of antigen presenting cells and the mechanism of Hsp70-mediated cross-presentation.

Hsp70 was shown to interact with the surface of antigen presenting cells but not with non-immune cells. This binding was dependent on the nucleotide state of the chaperone. Only the peptide-loaded, ADP-conformation of the molecule showed the described cell-surface interaction, while the empty, ATP-loaded state did not.

Previously published receptors for Hsp70 interaction with antigen presenting cells were critically evaluated and shown not to play a considerable role in this interaction.

The specific binding of Hsp70 was shown to be conferred by at least two distict interactions, namely by the N-terminal ATPase domain in one case and the C-terminal substrate binding domain in the other case.

The interaction of Hsp70 with the APCs was shown to lead to cross-presentation of chaperoned peptide on the cells' MHC class I molecules. Isolated ATPase and substrate binding domain of Hsp70 were analyzed for this function and the cross-presentation capabilities were mapped to the substrate binding domain.

Additionally, an attempt was made to understand the intracellular pathway of the Hsp70/peptide complex, and an assay was developed which allows analysis of the contribution of individual compartments employing dominant negative mutants of small GTPases.

II INTRODUCTION

II.1 Protein folding and molecular chaperones

There is no life imaginable without the various functions that proteins exert. Not only do they form the structural basis for all biologic units, but virtually all biological processes, from cell growth and division to differentiation and cell death, from nutrient uptake to metabolism, are coordinated by highly specialized protein networks.

In order to acquire their biologic activity, the common feature of all proteins is the necessity to obtain their native, functional state – one out of an overwhelming number of possible three-dimensional conformations which will render the protein biologically active: the protein needs to be folded. Incorrect folding, or the inability of certain proteins to remain correctly folded, results in a wide variety of pathological conditions such as Alzheimer's Disease, Chorea Huntington and cystic fibrosis, to name only a few.

II.1.1 Protein folding *in vitro* and *in vivo*

Since Anfinsen's pioneering experiments in the 1960's and 1970's, it has been well known that all information necessary to determine the native structure of a protein lies in its primary structure – the amino acid sequence. (Anfinsen, 1973). These experiments were performed *in vitro*, where the process of protein folding can be followed experimentally by first denaturing, i.e. unfolding a protein, and then allowing it to re-fold spontaneously to its native state upon removal or dilution of the denaturing agent.

Astonishingly enough, many proteins are able to refold *in vitro* to their native structure, which in almost all cases corresponds to the thermodynamically most favorable conformation (Anfinsen, 1973; Dobson and Karplus, 1999). These refolding reactions are reversible and proceed with biologically relevant kinetics (Schechter et al., 1970).

But how do proteins so efficiently and quickly fold into their native state? It has been estimated that for a theoretical protein of 150 amino acids, approximately 10^{300} possible conformations exist, and it would take about 10^{11} years to sample all possible conformations at maximal rate (Dinner et al., 2000; Levinthal, 1969). This so called "Levinthal paradox" led to the conclusion that protein folding cannot occur through random, unbiased searching of all possible confirmations (Daggett and Fersht, 2003), but rather must be split into individual steps with stabilized folding intermediates.

Such experimental setups as have led to our understanding of protein folding are usually carried out in a dilute aqueous environment, which reduces unfavorable interactions and favors the thermodynamically most stable conformation of a polypeptide. The situation in the eukaryotic and prokaryotic cytosol differs strongly from the *in vitro* scenario. As a newly synthesized protein chain emerges from the ribosome, it is immediately confronted with multiple influences from the crowded cellular milieu. The concentration of macromolecules in the bacterial cytosol has been estimated to be as high as 300 mg/ml (Cayley et al., 1991; Zimmerman and Trach, 1991).

The resulting excluded volume effect leads to many non-productive interactions of the nascent polypeptide chain with itself as well as with other

macromolecules, and such interactions can complete strongly with the folding into the native state (Ellis, 2000).

Additionally, translation is a relatively slow, vectorial process (4-20 amino acids per second), while the rate of folding is much faster ($t_{1/2}$ < 1 s). As a result, not the entire newly synthesized polypeptide chain is available to be folded at once. Especially in eukaryotic cells, this leads to an additional complication of the folding process due to the prevalence of large, multi-domain proteins, which often need to fold cooperatively, with key structures being formed after complete release from the ribosome.

These complications of protein folding *in vivo* have been elegantly solved by nature through the employment of a whole family of proteins designed to ensure correct protein folding and prevent aggregation, as well as to rescue existing proteins from partial stress-induced denaturation (Frydman, 2001), the molecular chaperones.

II.1.2 Molecular chaperone systems

Molecular chaperones represent several different structural families but share common features (Ellis and Hartl, 1999). They do not actively fold their substrates, but rather assist the self-assembly of proteins by capturing nonnative structures and protecting them from misfolding and aggregation (Hartl and Hayer-Hartl, 2002). Most chaperones recognize hydrophobic residues and/or unstructured backbone regions in their substrates (Hartl and Hayer-Hartl 2002). Such structural features are typical of nonnative proteins and become buried upon correct folding. These functions of molecular chaperones are necessary not only to ensure correct folding of newly synthesized protein chains, but also during conditions of cellular stress, and many

chaperones have been found to be inducible through heat and other stress factors, leading to the terminology heat-shock proteins or stress proteins. More than 20 different families of chaperones are now known, and they are distributed ubiquitously in all three kingdoms of life (Agashe and Hartl, 2000). These chaperone families cooperate in the cell to build highly functional networks of folding and regulation. Figure 1 illustrates the main protein folding pathways in bacteria and eukaryotes.

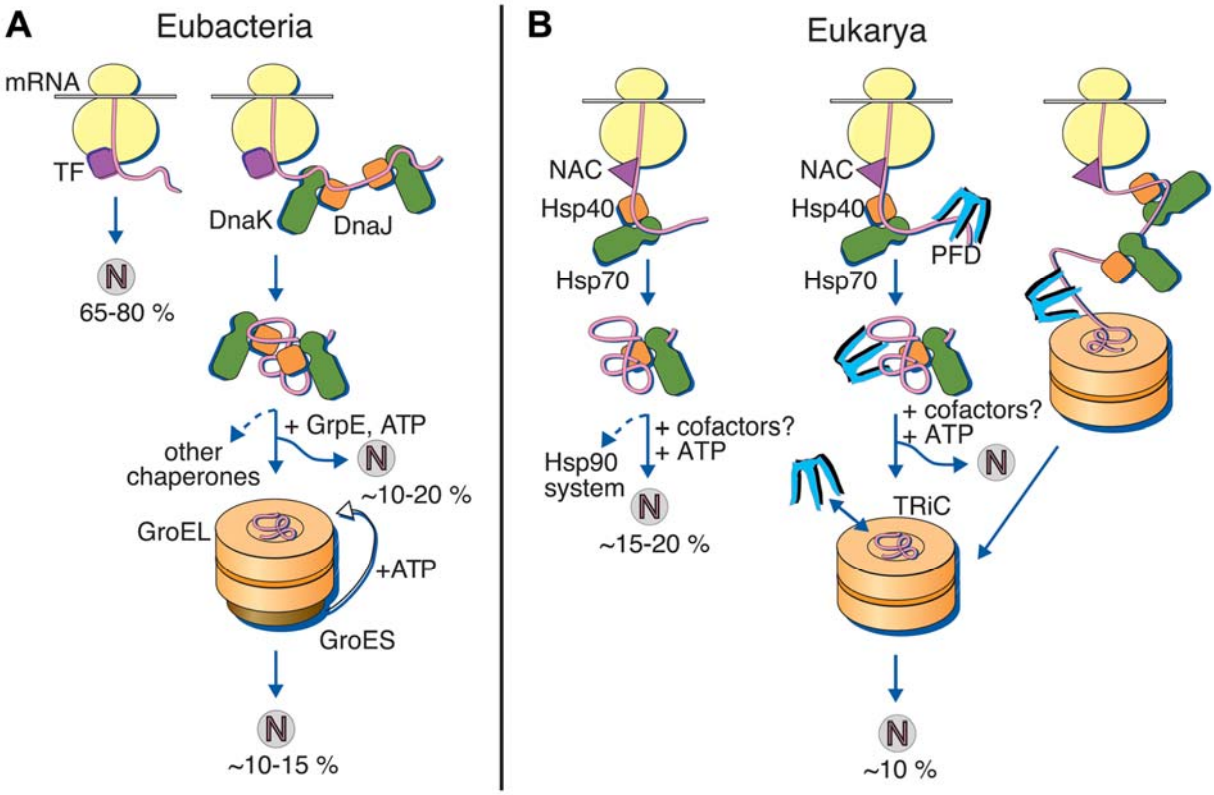

Figure 1: Model for chaperone-assisted de novo protein folding pathways in eukarya and bacteria.

N: Natively folded protein, TF: trigger factor, NAC: nascent chain-associated complex, PFD: prefoldin. **(A)** Bacterial proteins often fold spontaneously upon release from the ribosome and ribosome-bound TF. The remainder of proteins are assisted in folding by DnaK/J, which can further transfer substrates to the GroEL/ES system. **(B)** In the eukaryotic cytosol, NAC is thought to interact with nascent polypeptide chains, together with Hsp70/40. Some proteins are further transported to the Hsp90 system, while others are assisted by TRiC, possibly transferred by PFD. Adapted from (Hartl and Hayer-Hartl, 2002)

The first chaperones to interact with a nascent protein chain in bacterial cells are trigger factor and DnaK, the prokaryotic Hsp70 homolog. Trigger factor binds to the large ribosomal subunit as well as the nascent protein chain and is thought to provide a protective environment that shields hydrophobic stretches to keep them soluble. Active folding is then mediated by ATP-dependent chaperones, such as DnaK with its co-chaperone DnaJ and the GroEL/ES chaperonins (Young et al., 2004).

The eukaryotic cytosol lacks trigger factor, instead NAC (nascent chain associated complex) and, in yeast, RAC (ribosome-associated complex) are though to interact with the newly synthesized protein as it emerges from the ribosome.

The central component of the cellular chaperone network is formed by the large family of ubiquitous and highly conserved Hsp70 chaperones together with their Hsp40 co-chaperones and nucleotide exchange factors. Hsp70 chaperones are ATPases which support protein folding through active cycles of binding and release and additionally exert multiple other important functions in the cell. The 70 kDa family of molecular chaperones will be discussed in greater detail in the following chapter.

The chaperonins are a family of sequence-related proteins of about 60 kDa which associate into large, multimeric double-ring complexes that enclose a central cavity in each ring (Agashe and Hartl, 2000). Partially folded substrates are encapsulated in these central cavities, where folding can occur shielded from the cellular environment. Two classes of chaperonins can be distinguished. Group I chaperonins, represented by the bacterial GroEL as well as Hsp60 from mitochondria and chloroplasts, function in conjunction with a ring-shaped co-factor, GroES or Hsp10, which forms a lid on the folding cage (Frydman, 2001). In contrast, Group II chaperonins of the eukaryotic (and archaeal) cytosol do not interact with a lid-like co-

factor, possibly because the function of GroES is carried out by the chaperonin itself (Agashe and Hartl, 2000).

The Hsp90 family is a eukaryotic chaperone system that acts downstream of Hsp70/Hsp40 and binds a more restricted subset of substrate proteins in the cell (Nathan et al., 1997). It is highly specific for signal transduction molecules such as steroid hormone receptors and protein kinases, and is actively involved in the maturation of these signaling molecules (Smith, 2000).

An additional class of stress-inducible molecular chaperones is referred to as the small heat shock proteins. Eukaryotic representatives of this family, which bind unfolded proteins and prevent their aggregation are Hsp12, Hsp42 and the mammalian α-crystallin.

Other chaperones include the highly conserved Hsp100/Clp family members, which are often part of an ATP-dependant protein degradation system, as well as specialized chaperones in various cellular compartments, such as calreticulin and calnexin of the endoplasmic reticulum.

II.1.3 The Hsp70 family of chaperones

Hsp70 chaperones are monomeric, ~70 kDa proteins which are present not only in the bacterial, archaeal and eukaryotic cytosol, but also in the eukaryotic nucleus, mitochondria, chloroplasts and endoplasmic reticulum (Barral et al., 2004). In addition to their functions in *de novo* protein folding and prevention of aggregation, Hsp70 chaperones are involved in various other cellular processes such as protein targeting and membrane translocation, disassembly of oligomeric protein structures, protein degradation and control of regulatory protein activity (Bukau and Horwich, 1998; Mayer and Bukau, 2005). The cytosol of higher eukaryotes contains both constitutively expressed (Hsc70) and stress-inducible (Hsp70) members of this chaperone family, which exert their specialized functions together with their co-chaperones of the J-domain protein (Hsp40) family and nucleotide exchange factors.

Hsp70 chaperones consist of two major functional domains, a highly conserved N-terminal ATPase domain of 45 kDa and a less conserved C-terminal substrate-binding domain of 25 kDa. The substrate binding domain can be further subdivided into a β-sandwich domain of 15 kDa with a peptide binding cleft and a 10 kDa carboxy-terminal α-helical lid domain (Mayer and Bukau, 2005; Zhang and Zuiderweg, 2004).

The structures of both domains have been solved separately and recently the structure of a truncated bovine Hsc70 has been determined (Jiang et al., 2005). The ATPase domain of Hsp70 proteins consists of two lobes with a deep nucleotide binding cleft between them (Flaherty et al., 1990). This structure is highly conserved from bacteria (*E.coli*) to humans and shows striking similarity to actin and equivalent regions in hexokinase and glycerol kinase (Flaherty et al., 1990; Flaherty et al., 1991).

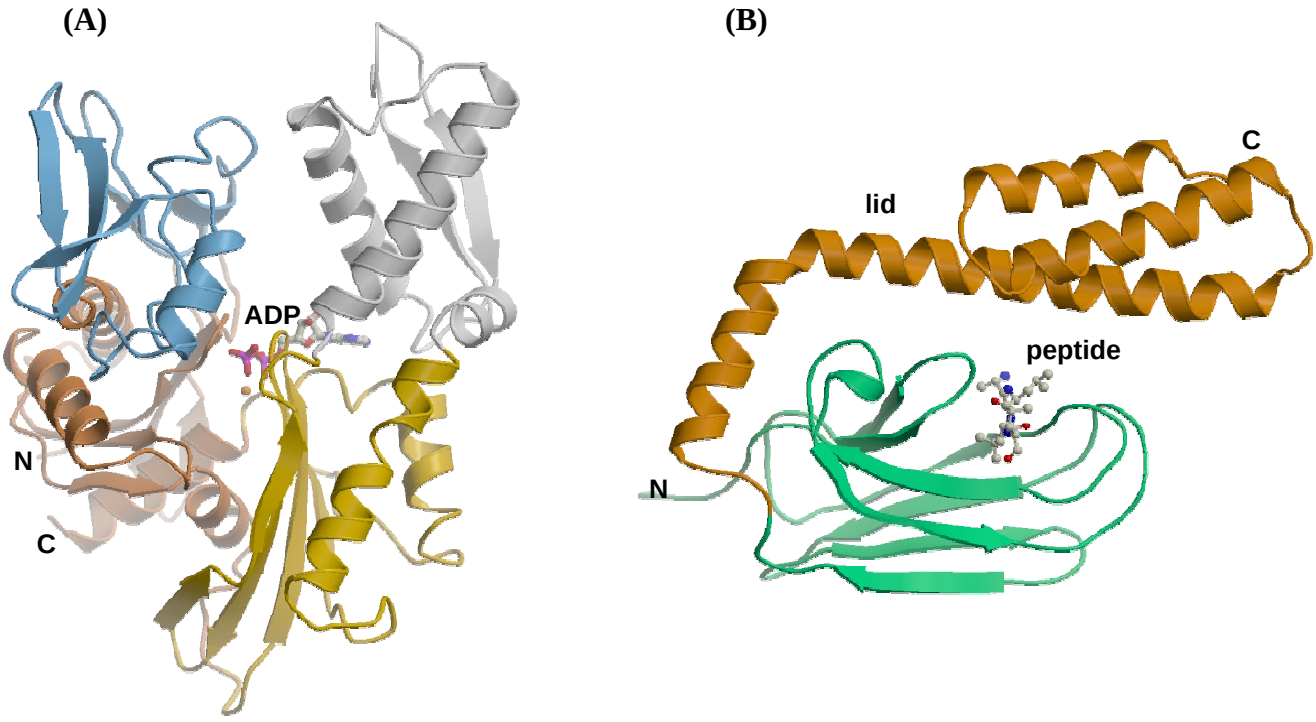

Figure 2: Structures of the domains of Hsp70

(A) The N-terminal ATPase domain of bovine Hsc70 with bound ADP (PDB code 3HSC). **(B)** The C-terminal substrate binding domain E.coli DnaK in complex with a peptide substrate (PDB code 1DKZ).

In vivo, the binding cycle of Hsp70/DnaK is assisted by co-chaperones of the Hsp40/DnaJ class and nucleotide exchange factors (GrpE for DnaK). Hsp40 chaperones themselves bind substrate and are thought to deliver unfolded polypeptide chains to the Hsp70s. The reaction cycle of Hsp70 proteins, best understood for the bacterial DnaK, is depicted in Figure 3.

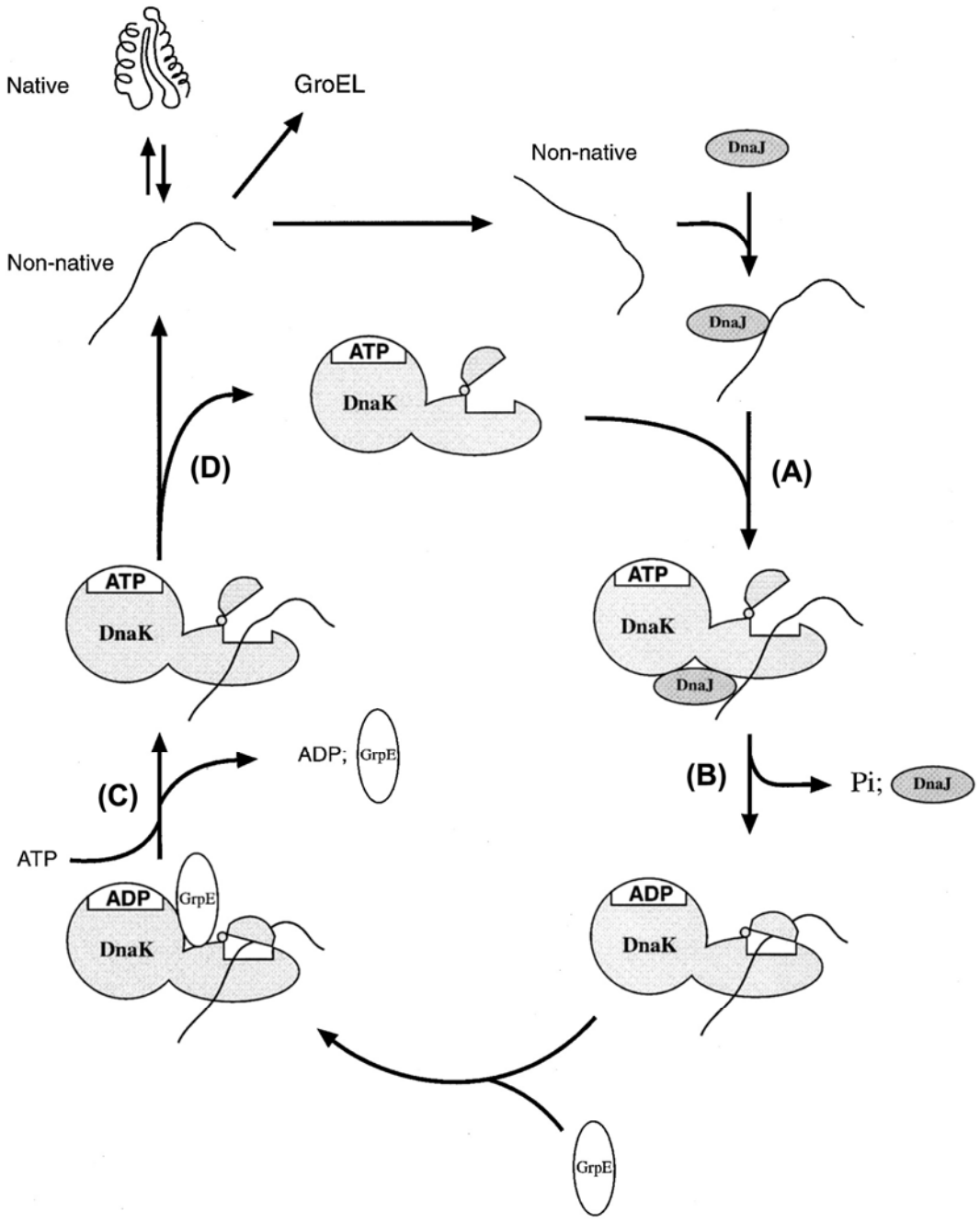

Figure 3: Model of the Hsp70 reaction cycle in polypeptide binding and release as exemplified by the bacterial DnaK, DnaJ and GrpE. Adapted from (Agashe and Hartl, 2000)

(A) DnaJ can bind to an unfolded polypeptide and then present it to the ATP-bound form of DnaK. **(B)** The stimulation of ATP hydrolysis resulting from the interaction of DnaK with both peptide substrate and DnaJ causes DnaK to rapidly attain its ADP-bound state that stably binds to the polypeptide substrate. **(C)** in *E. coli* and in mitochondria a second essential cofactor, GrpE, then catalyzes the exchange of the bound ADP for ATP. **(D)** This action reverts DnaK to its low substrate affinity state and facilitates the release of the substrate.

II.2 Innate and adaptive immune system

The mammalian immune system is commonly described as consisting of two interacting parts: the innate and the adaptive immune systems. Innate immunity provides a "first-line" of defense to invading pathogens. It includes physical barriers such as skin and mucous tissue as well as constitutively expressed anti-microbial molecules such as lysozyme and antimicrobial peptides. The cellular components of the innate immune system include phagocytic cells, the most common of which being macrophages, dendritic cells and neutrophils. A limited amount of cell-surface receptors recognize conserved microbial molecules, such as LPS, peptidoglycan and unmethylated DNA, and induce signal transduction pathways resulting in the secretion of various cytokines such as IL-12, CD40L, IL-1, IFN I and TNF, which facilitate an effective immune response.

Additionally, several classes of anti-microbial proteins can be activated upon encounter with a pathogen, e.g. the complement system, which, once active, recruits inflammatory cells, coats pathogens to make them more easily phagocytosed, and forms destructive pores in the surfaces of pathogens (Beutler et al, 2004).

While being highly effective against certain pathogens, the innate immune system fails to recognize many bacteria and viruses, which have evolved to escape this type of immune recognition e.g. by masking typical conserved surface molecules. Vertebrates have developed an additional set of protective mechanisms that encounter such pathogens, namely the adaptive immune system.

Adaptive, or acquired immune mechanisms are responsible for immunologic memory, i.e. the ability to mount a stronger and more effective immune response

against an antigen after having survived an initial encounter with it, thus resulting in long-lasting protection against certain pathogens.

Unlike innate immunity, adaptive immune responses are highly specific to the particular antigen that induced them. Specificity relies on the recognition of antigenic material by lymphocytes, the principal active components of the adaptive immune system. These cells represent a clonally diverse defense repertoire in which each lymphocyte bears multiple copies of a unique antigen receptor (Cooper and Alder, 2006), formed by somatic rearrangement on the DNA level.

B-Lymphocytes (bone marrow derived) recognize exposed determinants (epitopes) of intact molecules, including surface protein and carbohydrate moieties of invasive microbes (Cooper and Alder, 2006) and respond, once activated, with the secretion of antibodies specific for the encountered antigen.

T-Lymphocytes (thymus derived), on the other hand, recognize antigens only as fragments displayed by membrane bound molecules of the major histocompatability complex (MHC) in a process termed antigen presentation. The $CD8^+$ subset of T-cells recognizes protein antigens as peptides presented by molecules of the class I MHC, and can directly destroy cells infected with viruses (cytotoxic T-cells). $CD4^+$ T-cells, on the other hand, recognize peptides complexed to MHC class II molecules, and act indirectly by activating B lymphocytes to produce antibodies and macrophages to destroy bacteria (Robertson, 1998) (helper T-cells).

MHC class I and class II molecules are highly polymorphic protein complexes, with the variable amino acids being concentrated in the peptide binding groove. This polymorphism, together with the co-expression of MHC molecules from multiple genes, enables the MHC molecules of any given individual to bind a wide range of

peptides, which can then be efficiently recognized by the highly variable antigen receptors of T-Lymphocytes (Robertson, 1998).

II.2.1 MHC class I antigen presentation

MHC class I molecules of higher organisms are expressed on the surface of virtually all nucleated cells (Pamer and Cresswell, 1998). They facilitate the immunosurveillance of processes within these cells through continuous display of peptide fragments derived from intracellular polypeptides, thus alarming the immune system in case of viral infection or oncogenic transformation.

Mature, membrane associated MHC class I molecules consist of three noncovalently linked components (Figure 4). The heavy chain (α-chain), a polymorphic product of one of the MHC class I genes (Pamer and Cresswell, 1998), is a membrane-spanning glycoprotein and forms the binding groove for a short peptide of 8-10 amino acids. The associated light chain (β_2-microglobulin, β_2m) is a small soluble protein encoded outside the MHC locus (York and Rock, 1996). The peptide binding groove formed by the α1 and α2 domains is generally long enough to harbor a peptide of 8 or 9 amino acids in extended conformation, but longer peptides can also fit by bulging partly out of the groove.

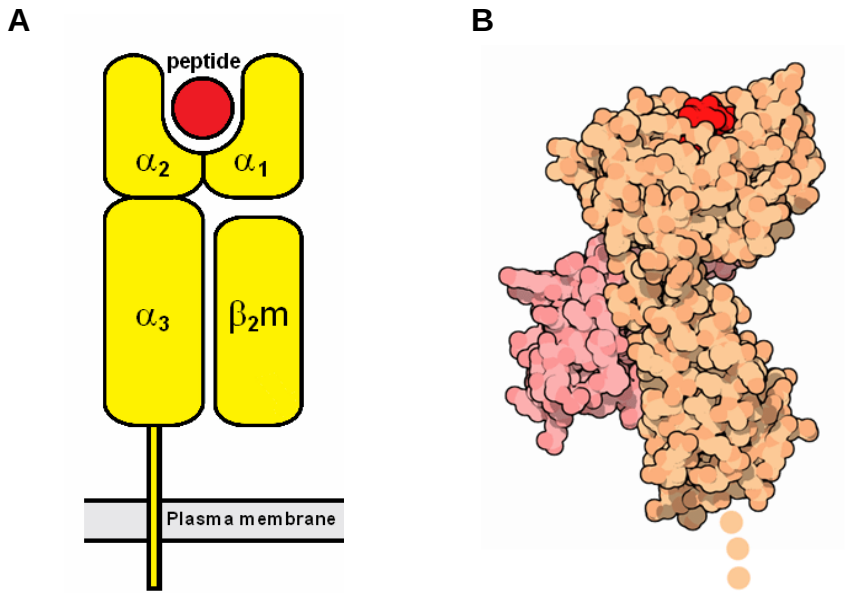

Figure 4: Composition and structure of MHC class I molecules
(A) Model of a typical MHC class I molecule (adapted from (Janeway, 1997)). **(B)** Murine H-2k(b) with SIINFEKL peptide, PDB code 1VAC (from http://www.rcsb.org).

The displayed peptides are derived, in the classical pathway of MHC class I antigen presentation, from intracellular proteins which have been processed by the proteasome (Figure 5). The proteasomal products (or peptides generated by other means) are then translocated into the endoplasmic reticulum (ER) by the transporter associated with antigen processing (TAP), a member of the ATP-binding cassette (ABC) transporter family, where they can be further trimmed by resident peptidases (Serwold et al., 2001).

Antigenic peptides in the ER are loaded on pre-assembled MHC class I – β_2m heterodimers, which are then released from TAP and transported to the cell surface, where they can be recognized by surveilling lymphocytes.

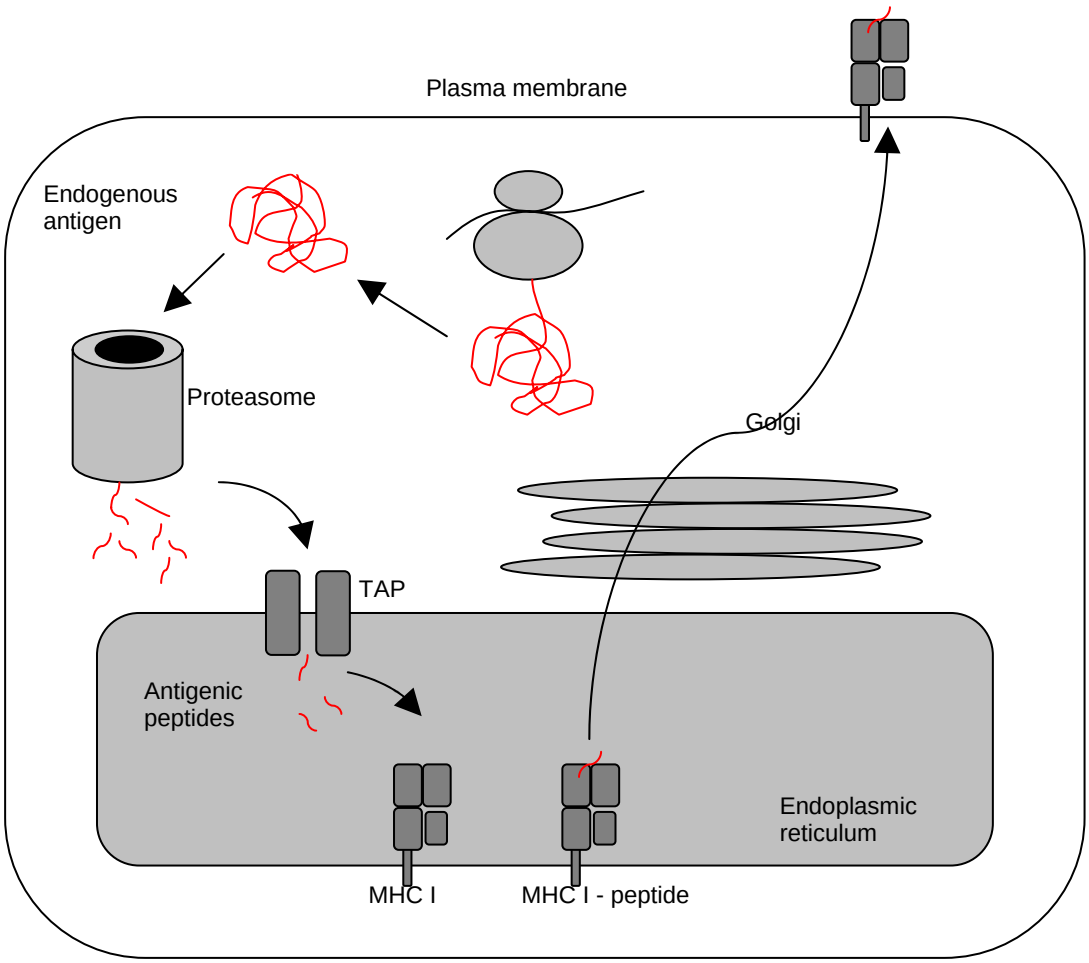

Figure 5: Assembly of MHC class I molecules

This mechanism, by which most cells display peptides derived from genes expressed within the cell, allows the $CD8^+$ T cells of the immune system to identify cells that are synthesizing abnormal genes (viral or mutant) and eliminate them (Rock and Shen, 2005).

II.2.2 MHC class II antigen presentation

Unlike intracellular antigens, peptides derived from extracellular pathogens are presented mainly by a specialized subset of immune cells, the MHC class II positive professional antigen presenting cells (pAPCs). B-cells, macrophages and dendritic cells belong to this cell group and possess a machinery specialized on uptake and processing of extracellular material and its presentation on MHC class II molecules.

MHC class II molecules are heterodimers composed of membrane-anchored α and β chains (Madden, 1995), the peptide binding groove being formed by the α1 and β1 domains (Figure 6).

Class II bound peptides can extend beyond class I bound peptides at both the N- and C-termini (Madden, 1995), with nine residues being bound within the class II groove and additional amino- and carboxy-terminal freedom (Jones, 1997).

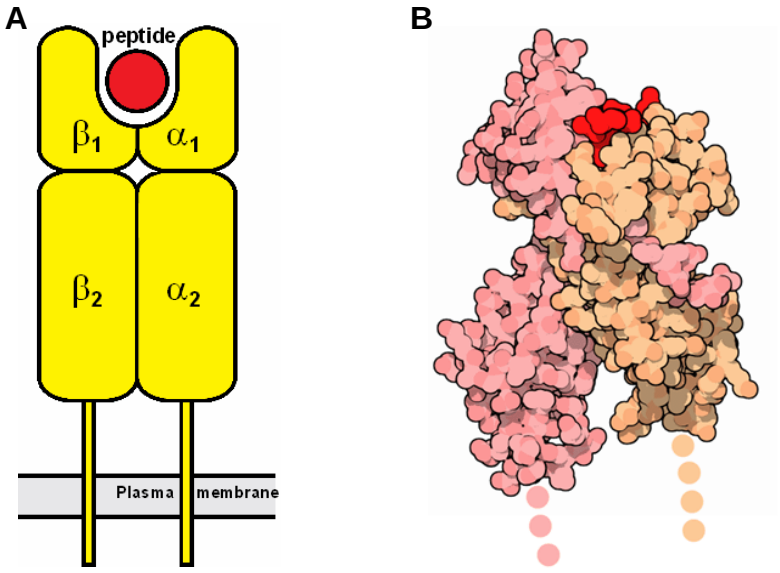

Figure 6: The structure of MHC class II molecules
(A) Model of a typical MHC class II molecule (adapted from (Janeway, 1997)). **(B)** structure of an MHC class II molecule displaying a peptide, PDB code 2IAD (from http://www.rcsb.org).

The MHC class II αβ heterodimers assemble in the ER with a third molecule, the invariant chain (Ii), which serves both as a targeting subunit and stabilizer of the peptide binding site (Watts, 1997). After transport to the trans-golgi network, the MHC class II-Ii complexes are targeted to vesicles within the endocytic pathway due to sorting signals present in the Ii cytoplasmic tail. In such vesicles, Ii is degraded, leaving a fragment of it in the peptide binding cleft of the MHC class II complex (Bryant and Ploegh, 2004). Peptide loading then occurs in late endocytic vesicles, in which epitopes are generated through proteolytic cleavage of endocytosed material. Loaded MHC class II molecules are subsequently transported to the cell surface (Watts, 2004).

II.2.3 Antigen cross-presentation

The presence of the appropriate MHC-peptide complex on the surface of virally infected or tumor cells is sufficient to engage committed $CD8^+$ killer T cells to destroy them (Ploegh, 2004). However, naïve $CD8^+$ cells must first be stimulated (primed) by antigen presented on the MHC class I molecules of professional antigen-presenting cells before they can clonally expand and destroy cells synthesizing foreign or mutant antigen. DCs and possibly some macrophages (Brode and Macary, 2004) are the only pAPCs that are capable of priming naïve $CD8^+$ (and $CD4^+$) cells: they express the necessary co-stimulatory molecules such as CD86, CD80 and CD40. A T-cell that receives both signals (antigen and co-stimulation) will become activated and produce cytokines, proliferate and differentiate into an effector cell.

But how does the immune system initiate a response against viruses that do not infect professional APCs or against malignant not-APC cells? Most immunologists now agree that the key is cross-presentation (cross priming) (Ploegh, 2004).

First described by Michael J. Bevan (Bevan, 1976a; Bevan, 1976b), cross-presentation is the process of uptake of exogenous material by APCs, followed by processing and subsequent presentation of the antigens on the cells' MHC class I molecules. *In vivo*, DCs acquire antigens from infected or transformed cells in the periphery, and then migrate to the lymph nodes where they display antigenic peptides in association with MHC class I molecules (Groothuis and Neefjes, 2005). Cross-priming describes the resulting stimulation of CTL responses.

Multiple routes for cross-presentation of antigenic material have been described in DCs and Mϕs. Internalization of exogenous antigens can be accomplished by endocytosis, pinocytosis, phagocytosis and macropinocytosis, the mode of uptake being primarily influenced by the character of the antigen. Even uptake of intracellular peptides through gap junctions has recently been proposed as an additional mechanism of antigen acquisition by cross-presenting cells (Neijssen et al., 2005).

A large number of studies performed on the mechanism of cross-presentation have revealed crucial roles for TAP and the proteasome, which would imply that the internalized antigens or peptides derived from them gain access to the cytosol of the cross-presenting cell (Figure 7). In this so-called "endosome-to-cytosol pathway", exogenous antigens can further follow the regular pathway of MHC class I antigen presentation (proteasome, TAP, ER), ultimately leading to their display on class I molecules of the antigen presenting cell.

Other reports have described an intersection between the recycling pathway of cellular class I molecules and endosomes/lysosomes which carry exogenous antigen (Gromme et al., 1999). In this model, antigens would be degraded by endocytic proteases, such as cathepsin S, rather than the proteasome (Groothuis and Neefjes, 2005), and subsequently become loaded on class I molecules within the endosomal compartment.

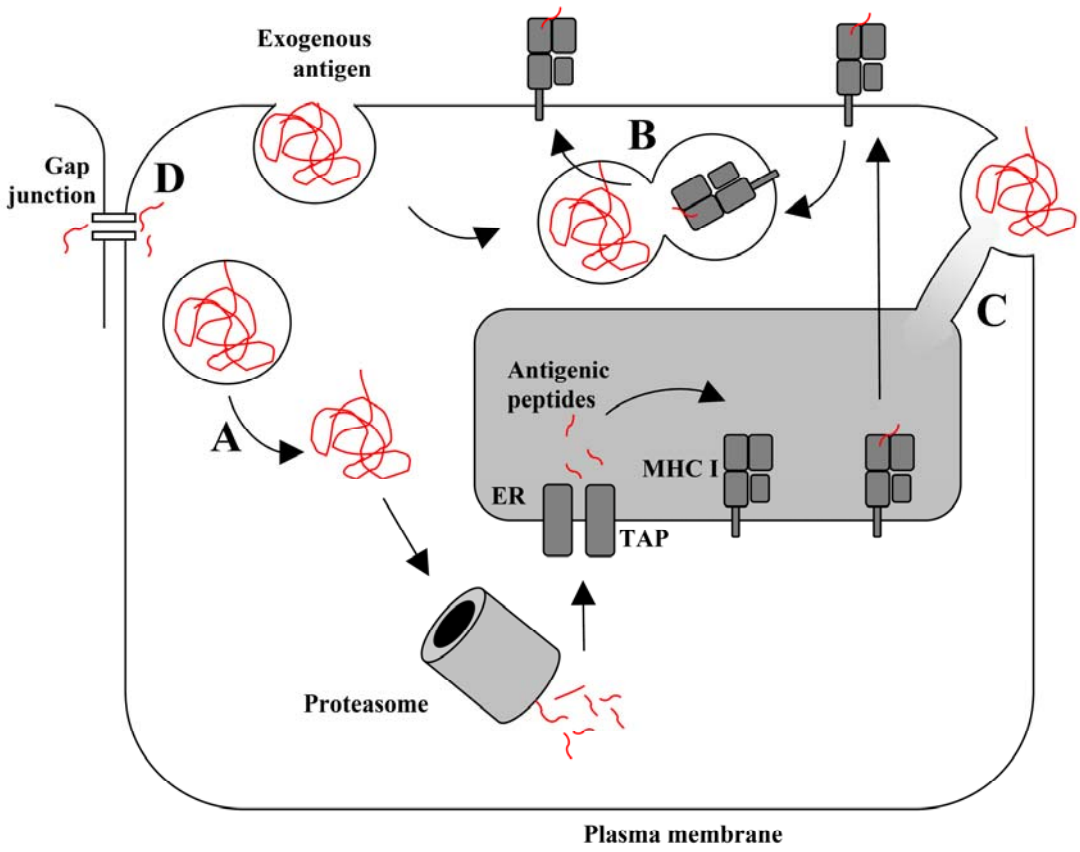

Figure 7: Possible pathways for MHC class I cross-presentation

(A) Endosome-to-cytosol pathway: Exogenous antigens gain access to the cytosol of the antigen presenting cell, from which they follow the classical MHC class I presentation pathway. **(B)** Recycling pathway: exogenous antigen carrying endosomes fuse with recycling MHC I – containing endosomes, where peptide load can be exchanged. **(C)** ER-phagosome model: ER contributes membrane to the phagosome. Exogenous antigen is exported into the cytosol through ERAD and back into the phagosome through TAP. **(D)** Gap junctions allow direct transfer of peptides from cancer/infected cells into the cytosol of pAPCs.

Recently, an alternative model for cross-presentation has become popular (Gagnon et al., 2002; Guermonprez et al., 2003; Houde et al., 2003). In the so-called "ER-phagosome model", the endoplasmic reticulum is thought to contribute membrane for the formation of phagosomes. Endocytic vesicles formed by this mechanism then contain the complete ER-based machinery necessary for MHC class I antigen presentation, including the Sec61 pore complex necessary for translocation of antigens into the cytosol. This model offers a new mechanistic explanation for MHC class I cross-presentation, but has recently been questioned (Groothuis and Neefjes, 2005; Touret et al., 2005).

Taken together, there is no clearly defined pathway known, until now, for antigen cross-presentation, but it rather seems that the pathway taken is dependent on the nature of the antigen. However, it is now clear that cross-presentation is a major mechanism for the immune surveillance of tissues. Importantly, *in vivo*, cross-presentation alone is not sufficient to initiate a protective immune response against the antigen. A second stimulus, leading to activation and maturation of the pAPC, is necessary to establish immunity rather than tolerance. Such a signal can be provided by adjuvants of bacterial origin, or, as recently described, by certain members of the heat shock family of proteins.

II.3 HSPs as key players of the immune system

In addition to the well-established role of molecular chaperones and heat shock proteins in the folding and re-folding of newly synthesized or misfolded proteins, the participation of HSPs in processes of the immune system has drawn much attention from researchers in the past years.

Directly related to the chaperoning properties of heat shock proteins is their participation in the intracellular events that lead to antigen presentation, such as degradation of proteins by the proteasome, transport to the endoplasmic reticulum, and chaperoning of MHC-antigen complexes within the ER and the secretory pathway.

Additionally, some chaperones have been found to serve as strong mediators of innate immune responses. The interaction of HSPs with cell surface receptors on antigen presenting cells has been shown to result in the secretion of inflammatory cytokines and nitric oxide by macrophages and dendritic cells, chemokines by macrophages, up-regulation of maturation markers on DCs and migration of DCs to draining lymph nodes (Binder et al., 2004). By virtue of these properties, Hsp70 has been clinically employed as an adjuvant in combination with other treatments to activate APC and break tolerance to tumor-associated antigens (Calderwood et al., 2005a; Srivastava, 2002a)

Of high medical relevance and the key basis of this study is the role that has been attributed to heat shock proteins in tumor rejection, which will be described in more detail in the following chapter.

II.3.1 Role of Hsp70 in anti-tumor immunity

The idea that a possible treatment or prevention of cancer could be provided through immunization dates back to the 1940s and 50s when several groups (Gross, 1943; Prehn and Main, 1957) reported that tumors could be immunogenic in syngeneic mice (Klein, 2001). Later, the experiments of Klein et al. (Klein et al., 1960) demonstrated the extraordinary phenomenon that one could immunize against syngeneic tumors in the same manner as one could against smallpox and polio viruses (Srivastava, 2002a). In contrast to viruses, the tumors were of self origin and yet were immunogenic. As the individual tumors were not cross-reactive, it was concluded that the tumor-specific antigens were individually rather than commonly tumor-specific (Srivastava, 2002a).

Following these exciting discoveries, a laborious search for the tumor-specific antigens began, revealing, with a few exceptions, molecules that were generally shared between cancers and normal tissues but no truly cancer-specific molecules (Binder et al., 2001).

In the 1980s, DuBois et al. (DuBois et al., 1982) and later Pramod Srivastava and co-workers (DeLeo and Srivastava, 1985; Palladino et al., 1987; Srivastava et al., 1986) finally managed to identify tumor rejection molecules common to cancers of divers origins. Surprisingly, all the well-characterized molecules described by this group turned out to be heat shock proteins of the hsp90, hsp70, calreticulin, or the grp170 family (Basu and Srivastava, 2000). They were able to show that homogeneous preparations of certain HSPs which were isolated from cancer cells elicited immunity to subsequent challenges with the cancer of origin, while corresponding preparations from normal cells did not (Srivastava, 2002b).

As the HSP preparations used to immunize were homogenous by all criteria tested, the idea arose that low molecular weight substances, at that time not detectable by polyacrylamide gel electrophoresis, were associated with HSPs and responsible for the specific immunogenicity of HSP preparations (Srivastava and Amato, 2001; Srivastava and Heike, 1991; Srivastava and Maki, 1991). This was later proven when the same group showed that depletion of peptides from tumor-protective Hsp70 through treatment of an immunogenic Hsp70 population with ATP rendered them ineffective in immunizing against cancer cells (Udono and Srivastava, 1993).

The association of Hsp70 (and gp96) with antigenic peptides was later shown *in vivo*, ruling out speculations that the observed association of Hsp70 and gp96 with antigenic peptides could be the result of an artifact occurring after cell lysis and during purification of the HSPs (Menoret et al., 1999).

Importantly, immunogenic HSP-peptide complexes can also be generated *in vitro* – Blachere et al. reconstituted Hsp70-peptide complexes and showed that while HSPs alone and peptides alone were not immunogenic, HSP-peptide complexes elicited MHC class I restricted, antigen-specific $CD8^+$ CTL responses (Blachere et al., 1997). Blachere et al. also demonstrated that the quantity of peptide complexed to HSP molecules required for successful immunization is extremely small - as little as a few hundred picograms of peptide, if complexed to HSPs, were found to be sufficient to immunize. These properties make HSPs powerful adjuvants for the generation of $CD8^+$ responses and the first adjuvants of mammalian origin (Srivastava, 2002b).

Tumor-derived HSP-peptide complexes as well as artificially reconstituted HSP-peptide complexes (Hsp70 and gp96) are now finding increasing and effective use in tumor immunotherapy (Calderwood et al., 2005b). Similarly, potent anti-viral

responses have been generated through the administration of HSP-peptide complexes. HSP-based tumor and antiviral vaccines have been highly successful in animal models and are currently undergoing testing in phase I, II and III clinical trials.

II.3.2 Hsp70 as a chaperone of adaptive immunity

The mechanism through which Hsp70-peptide complexes elicit anti-tumor and anti-viral responses has been delineated in some detail (Binder et al., 2004). Hsp70 is thought to become associated with tumor or viral antigens after their release into the cytoplasm by the proteasome. A number of studies have addressed the peptide sequence binding preferences of HSP70 proteins and indicated a similar peptide binding preference for MHC class I, e.g. the participation of hydrophobic and basic amino acids (Calderwood et al., 2005b; Flynn et al., 1989; Fourie et al., 1994; Gragerov and Gottesman, 1994; Gragerov et al., 1994; Wu and Wang, 1999).

Heat shock protein levels are increased during stress, and Hsp70 expression becomes deregulated in many types of cancer, resulting in elevated Hsp70 levels under non-stress conditions. As shown by Basu and co-workers (Basu et al., 2000), chaperone-peptide complexes get released during necrotic (but not apoptotic) cell death as occurs in cancers and virally infected cells, and the terminal stages of necrosis may favor antigen binding by Hsp70, since intracellular ATP levels decline and peptides become locked onto the ADP-associated HSP70 (Calderwood, 2005; Calderwood et al., 2005b). Indeed, a number of pathological conditions result in an increased Hsp70 level in the blood stream and the production of anti-Hsp70 antibodies. Hsp70 molecules, when released into the blood stream from necrotic cells, carry with

them a repertoire of peptides from their cell of origin, including peptides derived from mutated or re-expressed embryonic or developmental antigens.

The extremely low quantity of HSP-complexed peptide needed for immunization suggested that these molecules were doing something more than simply protecting the peptides from degradation or other such physical dangers, and the general biological principle that extraordinary efficiencies are often achieved through specific receptors was soon proven in this case (Srivastava, 2002a).

Multiple studies on Hsp70, gp96 and other chaperones have shown specific, saturable and competitive binding of these molecules to the cell surface of various cell types, most of them being professional antigen presenting cells (Arnold-Schild et al., 1999; Castellino et al., 2000; Lipsker et al., 2002; Singh-Jasuja et al., 2000; Sondermann et al., 2000). The specific binding of HSP-peptide complexes to antigen presenting cells is supported by the fact that the priming of immune responses by HSP-peptide complexes is exquisitely sensitive to abrogation of function of antigen presenting cells (Srivastava, 2002a).

The binding of HSP-peptide complexes to the surface of APCs leads to their receptor-mediated endocytosis (Arnold-Schild et al., 1999; Castellino et al., 2000; Singh-Jasuja et al., 2000; Wassenberg et al., 1999), followed by processing (if necessary) and cross-presentation of the chaperoned peptides on MHC class I molecules of the antigen presenting cell. This re-presentation of antigenic peptides, together with the adjuvant function of heat shock proteins, ultimately results in the described potent immune response.

So far, a number of cell surface receptors for HSPs have been proposed, including uptake-receptors as well as such responsible for the stimulation of innate immune mechanisms by HSPs. CD91, a molecule also referred to as the LRP/α_2-macroglobulin receptor, has been proposed as an uptake receptor first for gp96, and later also for Hsp70, Hsp90 and calreticulin (Basu et al., 2001; Binder et al., 2000). The ability of CD91 to interact with these proteins although they share no structural similarity reflects the promiscuity of the receptor - at least 32 ligands have been published to date (Binder et al., 2004) - and raises concerns about the specificity of the determined interactions. CD40, previously described as a signaling receptor, was described to be an uptake-receptor for mycobacterial (Wang et al., 2001) and mammalian Hsp70 (Becker et al., 2002).

A third candidate for the receptor-mediated endocytosis of Hsp70 was described by Delneste and colleagues, who identified LOX-1, a member of the same scavenger superfamily as CD91, as an additional receptor for endocytic uptake of Hsp70 and chaperoned peptides by human DCs (Binder et al., 2004; Delneste et al., 2002).

It is not yet well understood how Hsp70 interacts with cell-surface receptors on the molecular basis or which pathway within the cells is taken, ultimately leading to cross-presentation of chaperoned peptide. As previously mentioned, one study (Becker et al., 2002) described different binding affinities of Hsp70 to the surface of macrophages in dependence on the nucleotide state of the NBD, implicating a role of this domain in the interaction with the putative receptor CD40. Recently, another report, albeit on mycobacterial and not mammalian Hsp70, suggested that the C-terminal substrate binding domain is necessary for interaction with APCs leading to cross-presentation (MacAry et al., 2004). The same group hat previously shown that

the C-terminal portion of Hsp70 (aa359-610) stimulates cytokine production and DC maturation (Lehner et al., 2004).

The pathway Hsp70 and its chaperoned peptide take within the cell is also not clearly defined. For Hsc70 it has been demonstrated that this pathway is dependent on the nature of the antigen and the requirement for further processing (Castellino et al., 2000; Singh-Jasuja et al., 2001). If C-terminal processing of the peptide for transfer onto MHC molecules is required, re-presentation is dependent on the activity of the proteasome and TAP. If C-terminal processing is not required, transfer of peptide from HSP to MHC can occur inside the endosome as confirmed by confocal microscopy. The involvement of individual endocytotic compartments such as described for the uptake and processing of other molecules has not been characterized until now.

II.4 Aim of the project

The main focus of the project was to further delineate the mechanism by which Hsp70 chaperones peptides on the cross-presentation pathway. For a better understanding of this process, it was first necessary to investigate how the Hsp70 molecule binds to the surface of the antigen presenting cell. Given the multiple roles of described for Hsp70 as a chaperone and an adjuvant, it was expected that multiple cell surface receptors exist. The interaction between Hsp70 and previously described (uptake) receptors was to be critically evaluated.

The question which domains of Hsp70 the molecule interact with the putative receptors was to be accessed, with emphasis laid on the cross-presenting ability of the chaperone. An assay was to be established which would allow to differentiate between interactions between Hsp70 and the cell surface which lead to cross-presentation of chaperoned peptide and such that do not.

Additionally, an assay was to be established that would allow the application of dominant-negative mutants of proteins participating in endocytotic events, thus allowing targeted intervention in intracellular processes leading to cross-presentation of Hsp70-bound peptides.

III MATERIALS AND METHODS

III.1 Materials

III.1.1 Chemicals

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) and were of "pro analysi" grade.

Amersham Pharmacia Biotech (Freiburg, Germany):
γ-^{32}P-ATP

Biochrom KG Seromed (Berlin, Germany):
DMEM
VLE-RPMI 1640 medium
fetal calf serum (low endotoxin)
L-glutamine, penicillin/streptomycin

BioMol (Hamburg, Germany):
IPTG
HEPES

BioRad (München, Germany):
ethidium bromide
Bradford Protein Assay

BMA (Rockland, USA):
AgaroseLE

Calbiochem (Darmstadt, Germany):
Chlorophenolred-β-D-galactopyroanoside

Difco (Heidelberg, Germany):
Bacto Agar
Bacto Peptone
Bacto Tryptone
Bacto Yeast Extract

Fluka (Deisenhofen, Germany):
ADP
Dimethyle sulfoxide (DMSO)

Invitrogen (Karlsruhe, Germany):
2-mercaptoethanol

Merck (Darmstadt, Germany):
ampicillin
2-mercaptoethanol
EDTA

Molecular Probes (Leiden, Netherlands):
Alexa 488 protein labeling kit

National Diagnostic (Hessle, England):
Protogel (30 % Acrylamide, 0.8 % Bis-acrylamide)

New England Biolabs (Frankfurt a.M., Germany):
DNA ladder 100bp/1 kb
restriction endonucleases and buffers
T4 DNA ligase

Pierce (Rockford, USA):
BCA Protein Kit
Coomassie Plus Protein Kit

Promega (Mannheim, Germany)
Wizard®PlusSV Miniprep Kit

Qiagen (Hilden, Germany):

QIAprep Spin Mini prep kit

QIAquick PCR purification and gel extraction kits

QIAPrep EndoFree Plasmid Maxi Kit

Roche (Basel, Switzerland):

Ampicillin

Complete protease inhibitor

Dithiotreitol (DTT)

EDTA-free protease inhibitor

Shrimp Alkaline Phosphatase

Roth (Karlsruhe, Germany):

polyacrylamide/bisacrylamide solution 40 % (37.5 : 1)

Serva (Heidelberg, Germany):

Coomassie Serva Blue R

Stratagene (La Jolla, USA):

PfuTurbo™ DNA Polymerase

Herculase™ DNA Polymerase

QuickChange™ site-directed mutagenisis kit

III.1.2 Materials and Instruments

Abimed (Langenfeld, Germany):
Gilson Pipetman 2, 10, 20, 100, 200 and 1000 µl

Amersham Pharmacia Biotech (Freiburg, Germany):
EPS 300 electrophoresis power supply
prepacked chromatography columns:
HiTrap chelating 1ml and 5ml
HiPrep Desalting 5 ml
NAP-5 Sephadex G25
HiTrap Protein G 1ml

Amicon (Beverly, MA, USA):
concentration chambers (Centriprep, Centricon)
vacuum filtration unit (0,2µm)

Avestin (Mannheim, Germany):
EmulsiFlex C5 homogenizer

BD Biosciences (Heidelberg, Germany):
FACSCalibur
Cell culture plasticware

Beckman (Munich, Germany):
DU 640 UV/VIS Spectrophotometer
Avanti J-25 centrifuge with rotors JLA 10.500 and JA 25.50
Optima LE 80k ultracentrifuge with rotors SW28 Ti, SW 41 Ti, SW 55 Ti

Biometra (Göttingen, Germany):
T3 PCR-Thermocycler

BioRad (München, Germany):
MiniProtean 2 electrophoresis chamber
Gene Pulser Xcell electroporation device with Gene Pulser electroporation cuvettes

Bio-Tek (Bad Friedrichshall, Germany)
Synergy HT plate reader

Branson Ultrasonics (Danbury, CT, USA):
xxxsonifier

Corning Inc (Schiphol-Rijk, the Netherlands):
sterile cell culture pipettes 1 ml, 5 ml, 10 ml, 25 ml

Eppendorf (Hamburg, Germany):
centrifuges 5415C and 5417R, Thermomixer Comfort

Fisher Scientific (Schwerte, Germany):
Accumet Basic pH meter

Fuji (Tokyo, Japan) :
FLA 2000 Phosphoimager

Mettler Toledo (Gießen, Germany):
AG285 and PB602 balances

Millipore (Eschborn, Germany):
deionization system MilliQ plus PF
Millex-HA filters 0.22 µm
Steritop GP filter units 0.22µm

New Brunswick Scientific (Nürtingen, Germany):
Innova 4430 incubator

Perkin Elmer (Rodgau, Germany):
LS 50 B fluorescence spectrometer

Raytest (Straubenhardt, Germany):
AIDA version 2.31 gel imaging software

Savant (Strasbourg, France):
SGD 2000 slab gel dryer

III.1.3 Buffers and Media

All buffers and media were prepared with ddH_2O. Concentrations are given in weight per volume (w/v) for solid chemicals and in volume per volume (v/v) for liquids.

III.1.3.1 Buffers

ATP affinity buffer 1	50 mM Tris-HCl pH 7.5
	50 mM KCl
	5 mM $MgCl_2$
	(1 mM DTT)
	(10 % Glycerol)
ATP affinity buffer 2	50 mM Tris-HCl pH 7.5
	500 mM KCl
	5 mM $MgCl_2$
	(1 mM DTT)
	(10 % Glycerol)
ATPase Buffer	40 mM HEPES-KOH pH 7.4
	300 mM KCl
	10 mM $MgCl_2$
	2 mM DTT

Buffer Z	9 mM $MgCl_2$
	100mM 2-mercaptoethanol
	0.125 % Nonidet P-40 (NP-40)
	0.15 mM CPRG
	freshly dissolved in PBS
Denaturing wash buffer 1	50 mM Tris-HCl pH 7.5
	6 M guanidinium-HCl
Denaturing wash buffer 2	50 mM Tris-HCl pH 7.5
	6 M guanidinium-HCl
	20 mM imidazole
Denaturing wash buffer 3	50 mM Tris-HCl pH 7.5
	6 M guanidinium-HCl
	500 mM NaCl
Denaturing refolding buffer	20 mM Tris-HCl pH 7.5
	500 mM NaCl

Hanks' Balanced Salt Solution (HBSS)

with calcium and magnesium	1.26 mM $CaCl_2$
	0.493 mM $MgCl_2$
	0.407 mM $MgSO_4$
	5.33 mM KCl
	0.441 mM KH_2PO_4
	4.17 mM NaHCO3
	137.93 mM NaCl
	0.338 mM Na2HPO4
	5.56 mM D-Glucose
HSP binding buffer	10mM MOPS
	5mM $MgCl_2$
	150mM KCl
	pH adjusted to 7.2 with KOH
PBS	137 mM NaCl
	2.7 mM KCl
	4.3 mM Na_2HPO_4
	1.4 mM KH_2PO_4
	adjust pH to ~7,3 with HCl

PBG	137 mM NaCl
	1 mM KCl
	2 mM $MgCl_2$
	4,3 mM Na_2HPO_4
	1,4 mM KH_2PO_4
	adjust pH to ~7,3 with HCl
Protein G Binding Buffer	20 mM Sodium Phosphate buffer , pH 7
	100 mM NaCl
Protein G Elution Buffer	100 mM Glycin*HCl
	pH 2.7 adjusted with Tris pH 9
SDS-PAGE electrophoresis buffer	50 mM Tris-HCl pH 8.3
	380 mM glycine
	0.1 % (w/v) SDS
SDS-PAGE sample buffer (5x)	2 % SDS
	60 mM Tris-Cl pH 6.8
	24 % (v/v) glycerol
	0.5 % (w/v) bromphenol blue
	14.4 mM mercaptoethanol (added prior to use)

TAE-buffer
242 g/l Tris base
57.1 ml/l acetic acid
50 mM EDTA

Transformation Buffer (TB)
10 mM Pipes
55 mM $MnCl_2$
15 mM $CaCl_2$
250 mM KCl2
pH 6.7 – 7 (KOH), adjusted prior to $MnCl_2$ addition

TLC running buffer
0.5 M formic acid
0.5 M LiCl

III.1.3.2 Media

Unless stated otherwise, media were autoclaved after preparation.

LB-medium
10 g/l bacto tryptone
5 g/l bacto yeast extract
5 g/l NaCl
pH adjusted to 7.4 with NaOH

LB-agar
16 g/l bacto agar
dissolved in LB-medium

SOB-medium

20 g/l bacto tryptone

5 g/l bacto yeast extract

10 mM NaCl

2.5 mM KCl

10 mM MgCl2

10 mM MgSO4

SOC-medium

20 g/l bacto tryptone

5 g/l bacto yeast extract

0.5 g/l NaCl

After autoclaving, supplement with sterile-filtered

10 mM $MgCl_2$

10 mM $MgSO_4$

0.4% (w/v) Glucose

III.1.4 Bacterial strains and mammalian cell lines

Table 1: E.coli strains used in this study.

Designation	Reference
DH5α	Novagene
BL21 (DE3)	Novagene
SURE	Novagene
XL1 Blue	Stratagene

Table 2: Mammalian cell lines used in this study.

Designation	Organism	Tissue/ cell type	Growth properties	Properties	Reference
ANA-1	*Mus musculus* (mouse)	macrophage	adherent	Adherent murine macrophage cell line, H-2b	(Cox et al., 1989)
B3Z	*Mus musculus* (mouse)	T-lymphocyte hybrid	suspension	SIINFEKL/Kb specific, LacZ under the control of IL-2 enhancer	(Karttunen et al., 1992)
DC2.4	*Mus musculus* (mouse)	dendritic cells	adherent	H-2b	(Shen et al., 1997)
COS-7	*Cercopithecus aethiops* (African green monkey)	kidney/ fibroblast	adherent	SV40 transformed, T antigen	ATCC No.: CRL-1651
HEK 293T	*Homo sapiens* (human)	kidney/ epithelial	adherent	Highly transfectable derivative of the 293 cell line into which the gene for SV40 T-antigen was inserted.	ATCC No.: CRL-11268
Raji	*Homo sapiens* (human)	Haematopoietic B lymphocyte	suspension	Express high levels of CD40	ATCC No.: CCL-86
Raw 264.7	*Mus musculus* (mouse)	macrophage	adherent	H-2d	ATTC-No.: TIB-71
Raw 309 Cr.1	*Mus musculus* (mouse)	monocyte/ macrophage	adherent	H-2d, H-2b	ATCC No.: TIB-69

III.1.5 Plasmids

Table 3: Plasmids used in this study.

ORF	Vector	Restriction sites	Comments
Hsp70 human	pProEx HTa (Invitrogen)	Eco RI/Xho I	N-terminal His_6-purification tag, cleavable with TEV protease
N70 human		Bam HI/ Xho I	
C70 human		Bam HI/ Xho I	
Ova-Hsp70		Bam HI/ Xho I	
Ova-N70		Bam HI/Xho I	
H-2K(b) murine	pcDNA3.1 (Invitrogen)	Eco R V	Includes KOZAK sequence of K(b) gene
ARF 6 human	pCMV-Tag2A (Stratagene)	Eco RI	N-terminal FLAG tag
ARF 6 T27N			
ARF 6 Q67L			
CD40 human	pcDNA3	Nhe I/Xho I	
Dyn I human	pcDNA3.1 (Invitrogen)	Xho I/Xba I	Wild type
Dyn I K44E			Dominant negative mutant
Rab 5 human	pCX	Eco RI	Wild type
Rab 5 S34N			Dominant negative mutant
Rab 5 Q79L			Constitutively active mutant
Rab 7 human	pcDNA3.1	Hind III/Apa I	Wild type
Rab 7 T22N			Dominant negative mutant
Rab 7 N125I			Dominant negative mutant
Rab 7 Q67L			Constitutively active mutant

III.1.6 Antibodies

Table 4: Antibodies used in this study.

Antibody	Species	Reference
Primary antibodies		
CD40 human	mouse	Ancell
CD40 murine	hamster	BD Biosciences
H2-k(b)-Biotin	mouse	BD Biosciences
H2-k(b)-PE	mouse	BD Bioscience
25D1.16	mouse	(Pace et al., 1995)
LRP/α_2MR/CD91	mouse	Progen
Secondary antibodies and reagents		
FITC anti mouse	goat	Dianova
Streptavidin PE	-	BD Biosciences

III.2 Molecular biological methods

Unless stated otherwise, *E.coli* cells were grown at 37 °C with vigorous shaking (220rpm).

III.2.1 *E.coli* glycerol stocks

For long-term storage of bacteria, cells were grown to saturation over night in LB medium supplemented with appropriate antibiotics. 1.4 ml of culture were mixed with 0.6 ml sterile glycerol and transferred into cryovials for snap freezing in liquid nitrogen or dry ice/ethanol and storage at -80 °C.

III.2.2 Plasmid DNA purification, small scale

For purification of plasmid DNA, 5 ml LB medium supplemented with antibiotics were inoculated with a single *E. coli* colony harbouring the plasmid of interest and grown to saturation over night at 37 °C. Cells were harvested by centrifugation and DNA was extracted according to the manual of QIAprep Spin Miniprep Kit (Qiagen, Hilden, Germany) or Wizard®PlusSV Miniprep Kit (Promega, Mannheim, Germany).

III.2.3 Plasmid DNA purification, large scale

Plasmid DNA for transfection of mammalian cells was purified with the QIAPrep EndoFree Plasmid Maxi Kit (Qiagen, Hilden, Germany). Routinely, 250-500 ml of LB medium supplemented with appropriate antibiotics were inoculated with 5 ml over night culture grown to saturation. Cell pellets were harvested by centrifugation and plasmid DNA was extracted according to the manufacturer's instructions.

III.2.4 Optical density measurements

DNA concentration of purified plasmid DNA was measured by UV absorption spectroscopy at a wavelength of 260 nm, assuming that 1 A260 unit corresponds to 50 µg double stranded DNA in H_2O.

Optical density of *E.coli* cultures was determined at 600 nm.

III.2.5 DNA sequencing

Sequencing of plasmid DNA was performed by Sequiserve (Vaterstetten, Germany).

III.2.6 Restriction digestion and ligation of DNA

Fragmentation of DNA for analysis and cloning was performed by digestion of plasmid DNA or PCR products with restriction endonucleases. Enzymes and buffers were supplied by New England Biolabs, Frankfurt a.M., Germany and used according to the manufacturer's recommendations. Purified PCR products and vectors were digested 1 h – over night at 37 °C in a 50 µl reaction volume. Vectors were additionally de-phosphorylated with calf intestinal phosphates for 1 hour. DNA fragments were separated by preparative agarose gel electrophoresis and purified from gel slices using anion exchange chromatography (QIAquick Gel extraction kit, Qiagen) following the manufacturer's instructions.

Ligation reactions typically contained a molar insert to vector ration of at least 4:1 in a volume of 20 µl and were incubated over night at 16 °C.

III.2.7 DNA agarose gel electrophoresis

Separation of DNA fragments for analysis or preparation was performed using a Horizon 58 electrophoresis chamber (Whatman) with an EPS 300 power supply (Pharmacia Biotech) operated at constant voltage of 80 – 120 V. Gels contained 1-2 % agarose in TAE Buffer and 1 µg/ml ethidium bromide for visualization of double-stranded DNA.

III.2.8 Generation of chemically competent *E.coli*

E.coli competent for chemical transformation were generated by the method described in (Inoue et al., 1990). 250 ml SOB medium were inoculated from a starter culture grown over night in LB and cultured until an oD_{600} of approximately 0.6. Culture was chilled on ice for 10 min before harvesting the cells by centrifugation at 2500 x g, 4 °C. Cell pellets were gently resuspended in 20 ml ice-cold transformation buffer and DMSO was added to a final concentration of 7 %. The suspension was chilled on ice for 10 min and subsequently aliquoted, snap-frozen in dry-ice/ethanol or liquid nitrogen and stored at -80 °C. Competent cells generated by this method resulted in approximately $5*10^6$ CFU / µg plasmid DNA.

III.2.9 Chemical transformation of *E.coli*

For transformation, 50 µl competent cells were thawed on ice and gently mixed with 10-100 ng plasmid DNA or 1 – 10 µl ligation reaction. After 20 min on ice, the suspension was heat shocked at 42 °C for 45 – 60 s and placed on ice for 2 min. After addition of 1 ml SOC medium, cells were incubated at 37 °C for 1 h with shaking. The cell suspension was subsequently plated on selective plates and incubated at 37 °C over night.

III.2.10 Generation of electro-competent *E.coli*

For higher efficiency of transformation, *E.coli* cells were made competent for electroporation. A 5 ml over-night culture of LB medium was inoculated with a single colony from a freshly streaked plate. 2.5 ml of this culture were then used to inoculate 500 ml of LB medium, which was then allowed to grow until an oD_{600} ~ 0.6. Cells were harvested by centrifugation (20 min, 5000 x g, 2 °C) and washed twice with ice-cold, sterile ddH_2O (double pellet volume). Pellets were then resuspended in 10 % glycerol (pellet volume), aliquoted and frozen in a dry-ice/ethanol bath for storage at -80 °C.

III.2.11 Electroporation of *E.coli*

Electro-competent cells were thawed on ice and 50 µl cell suspension per reaction was gently mixed with 10-100 ng, but not more that 2 µl, DNA. After 1 min on ice, cells were transferred into pre-chilled 2 mm gap electroporation cuvettes. Electroporation was performed with the following settings: 200 Ω, 25 µF, 2.5 kV. The cells were immediately taken up in 1 ml SOC medium and incubated for 30-60 min at 37° with gentle shaking before being plated on agar plates.

III.3 Protein biochemical methods

III.3.1 Determination of protein concentration

Protein concentration was spectrophotometrically at OD_{595} by using Bradford reagent (Pierce, Rockford, USA) (Bradford, 1976) and a BSA standard dilution series or at OD_{280} by applying the Lambert-Beer equation and the theoretical extinction coefficient (Pace et al., 1995). Extinktion coefficients were determined using the ProtParam tool at the Expasy server (http://www.expasy.ch).

III.3.2 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analyzed by SDS PAGE under denaturing, reducing conditions (Laemmli, 1970). Samples were prepared by addition of 5x SDS PAGE sample buffer and heating to 96 °C for 5 min prior to loading onto the gel.

Stacking gels were made with 4 % and resolving gels contained 10 % or 12 % polyacrylamid (AA) (see table 5). Ammonium-peroxo-disulfate (APS) and N,N,N',N'-di-(dimethylamino)-ethane (TEMED) were adding immediately before casting the gels. Electrophoresis was carried out at constant current (40 mA – 150 mA per Gel) in electrophoresis buffer.

Table 5: Composition of stacking and separating gels for SDS-PAGE (10 ml).

	Resolving gel 12 % AA	**Resolving gel 10 % AA**	**Stacking gel 4 % AA**
30 % AA, 0.8 % bis-AA	4 ml	3.3 ml	1.3 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml	2.5 ml	-
0.5 M Tris-HCl, pH 6.8	-	-	2.5 ml
10 % SDS	0.1 ml	0.1 ml	0.1 ml
H_2O	3.4 ml	4.1 ml	6.1 ml
TEMED	5 µl	5 µl	10 µl
10 % APS	50 µl	50 µl	50µl

Following electrophoresis, gels were stained with Coomassie Staining Solution (0.1 % Coomassie Brilliant Blue R-250, 30 % methanol, 10 % acetic acid) and destained with 50 % methanol, 12 % acetic acid.

III.3.3 Purification of Hsp70, N70 and C70

Full-length Hsp70 as well as the individual domains N70 and C70 were purified as soluble, his-tagged proteins from *E.coli* BL21. Bacterial cells transformed with the respective plasmids were plated on LB^{Amp}-agar from glycerol stocks. Colonies were allowed to grow over night at 37 °C.

For the purification of full-length Hsp70, single colonies were picked and used to inoculate 1 l of LB^{Amp} medium, which was allowed to grow over night. The culture was then diluted into 5 l LB^{Amp}-medium and grown to an oD_{600} of 0.4-0.5. Protein expression was induced by addition of 1mM IPTG and allowed to proceed for 4 hours.

Bacterial cells were harvested by centrifugation for 20 min at 3000 x g and 4 °C. From this step onwards, all procedures were carried out on ice or at 4 °C (centrifugation steps) and with ice-cold material.

For lysis of bacterial cells, pellets were resuspended in 50 ml PBS, 0.1 % Triton and complete EDTA-free protease inhibitor (Roche, Basel, Switzerland). The suspension was homogenized by four passages through a French Press (EmulsiFlex C5) operated at 10000-15000 psi.

Removal of cell debris was achieved through ultracentrifugation at 100 000 g for 45 min and subsequent filtration (0.45µm). Salt content of the cleared lysate was adjusted by addition of NaCl and imidazole to a final concentration of 0.5 M and 10mM, respectively. Lysate was then applied to a 5 ml HiTrap chelating column, which had been previously loaded with 0.1 M $NiSO_4$, at a flow rate of 1 ml/min, powered by a peristaltic pump. The column was washed with 5 volumes of 50 mM Tris, pH 8.0, 0.5 M NaCl and bound His_6-tagged protein was eluted with 50 mM Tris, pH 8.0, 50 mM NaCl, 0.5 M imidazole. 15 1 ml fractions were collected and aliquots were subjected to SDS page for quality and quantity control. Fractions containing significant amounts of pure His-Hsp70 were pooled and dialyzed against 4 l of ATP affinity buffer 1 containing DTT and glycerol, followed by ATP affinity chromatography.

The purification of the amino-terminal and carboxy-terminal domains of Hsp70, as well as Ova-Hsp70 were performed in the same manner with following modifications.

N70 was expressed in a total culture volume of 1.1 l, and a 1 ml HiTrap chelating column was used. Only the first 2 ml of the eluate were collected since the following fractions contained heavily precipitated material.

C70 was expressed in a culture volume of 4 l and harvested 3 h after induction. Fractions containing the first 50 ml of eluate were pooled and dialyzed twice against 4

l of either HSP binding buffer or PBG, both supplemented with 10 % glycerol and 1 mM DTT. The purified protein was aliquoted and frozen in ethanol/dry ice.

Ova-Hsp70 was expressed in a culture volume of 1.1 l and ml 2-4 of the eluate were pooled and frozen after dialysis.

III.3.4 Purification of Ova-N70 from inclusion bodies

Ova-N70 was expressed as insoluble his-tagged protein in a culture volume of 1.1 l as described above. Cell pellets were resuspended in PBS supplemented with proteinase inhibitor (complete mini EDTA-free) and lysozyme (400 µg/ml) and incubated for 30 min with gentle agitation. Triton-X-100 was added to a final concentration of 1 % and cell suspension was subjected to three passages through the cell disrupter followed by five sonication steps. Three ultracentrifugation steps were carried out to isolate insoluble protein (30000g, 30 min, 4 °C). Final pellet was solubilized in 20 ml 6 M guanidinium-HCl, 50 mM Tris pH 7.5. The solubilized material was separated from cellular debris through ultracentrifugation (100000 g, 60 min, 4 °C) and an aliquot was subjected to SDS PAGE for quality and quantity control.

Approximately 10 mg of His_6-tagged protein were applied on a 1 ml HiTrap chelating column which had been loaded with $NiSO_4$ and equilibrated with 6 M guanidium-HCl, 50 mM Tris pH 7.5. The column was washed with five volumes each of denaturing wash buffer 1, 2 and 3, followed by refolding of bound protein with denaturing refolding buffer. After an additional washing step with PBS, protein was eluted with 200 mM imidazole/PBS. Fractions containing ml 3 – 7 of eluate were pooled and dialyzed over night against HSP binding buffer supplemented with DTT

and glycerol. Sample was concentrated five-fold and centrifuged at 17000 x g to remove residual precipitates prior to aliquoting and freezing in an ethanol/dry ice bath.

III.3.5 ATP affinity chromatography

Full-length Hsp70 as well as constructs containing the amino-terminal ATPase domain of the molecule were further purified through ATP affinity chromatography. For the generation of 2 mg ATP-agarose resin, 140 mg adenosin-5'-triphosphate-agarose (Sigma) were incubated in 10 ml ATP affinity buffer 1 for 30 – 60 min on ice. After removal of residual liquid, the resin was washed twice with buffer 1 and centrifuged briefly to settle the gel. Protein solution was applied to the resin and allowed to bind on ice for 30 – 60 min with occasional agitation. After removal of unbound material, resin was washed twice with ATP affinity buffer 1, once with buffer 2, and an additional time with buffer 1. Fractions of bound protein were eluted with 1 ml buffer 1 supplemented with 3 mM ATP for 5 min on ice and analyzed by SDS PAGE. Pooled fractions containing pure protein were dialyzed against PBG or Hsp binding buffer containing DTT and glycerol, aliquoted and flash frozen on dry ice/ethanol.

III.3.6 ATPase assay

ATPase assays were performed as described earlier (Obermann et al., 1998). ATPase activity was calculated from the amount of α-^{32}P-ATP hydrolyzed in a certain time period. Proteins of interest were preheated to 30 °C in ATPase buffer and reactions were started by addition of ATP supplemented with 1 µC α^{32}P-ATP. After 0, 5, 20, 40 and 60 min, aliquots were removed, reactions were stopped with 25 mM

EDTA pH 8.0 (final concentration), and flash frozen until further use. 1µl of each fraction was analyzed by thin layer chromatography (TLC) on PEI-cellulose plates (Merck) in TLC running buffer. Membranes were dried and exposed on detection plates (FuJi Film BAS-IP MS2040S). ATP and ADP spots were quantified using the Phosphoimager (FuJi Film FLA-2000) and the Aida software (Raytest).

III.3.7 Labeling with Alexa dyes

Hsp70 and N70 were both labeled with Alexa-488 C_5-maleimide (Molecular Probes). Typically, 1.5 mg of protein (2 mg/ml) were incubated with a 20 fold molar excess of reactive dye in PBS at 4 °C over night. Labeled protein was separated from free dye through extensive dialysis against ATP affinity buffer 1 before being subjected to ATP affinity chromatography. Degree of labeling was determined by measuring the absorption spectra and applying Lambert-Beer's equation as following:

$$\textit{Protein concentration (M)} = \frac{[A_{280} - A_{494} \times 0.11] \times \textit{dilution factor}}{E_{280}}$$

Where E_{280} is the molar extinction coefficient of the protein and 0.11 is a correction factor to account for absorption of the dye at 280nM.

$$\textit{moles dye per mole protein} = \frac{A_{494} \times \textit{dilution factor}}{71\,000 \times \textit{protein concentration (M)}}$$

Where 71 000 is the approximate molar extinction coefficient of the Alexa Fluor 488 dye at 494 nm.

III.3.8 Formation of Hsp70/Ova-BiP complexes

For loading of Hsp70 with Ova-BiP, 4.8 µM Hsp70 was incubated with 96 µM Ova-BiP in PBG supplemented with 100µM ATP in a final volume of 500 µl for 30 min at 25 °C. Peptide loaded Hsp70 was stabilized by addition of 1 mM ADP and incubation at 25 °C continued for 2 h. Alternatively, a quicker loading protocol of 15 min (ATP) and 25 min (ADP) at 37 °C resulted in similar efficiencies.

For separation of Hsp70/Ova-BiP complexes from free peptide, the 500 µl reactions were applied to Nap-5 gel filtration columns equilibrated with PBG. Complexes were eluted with 1 ml PBG, and drops 7 – 14, corresponding to µl 200 - 470, contained Hsp70/Ova-BiP complexes but not free peptide. Immediately after elution, Hsp70/Ova-BiP complexes were utilized for cross-presentation assays.

III.3.9 Formation of C70/Ova-BiP complexes

C70/Ova-BiP complexes were generated by incubation of 4.8 µM C70 with 96 µM Ova-BiP in PBG in a final volume of 500 µl over night at 25 °C.

Separation from free peptide was accomplished as described for Hsp70, with the distinction that drops 5 – 14 were collected.

III.3.10 Anisotropy measurements

Fluorescent anisotropy measurements were conducted in an LS 50 B fluorescence spectrometer (Perkin Elmer). For the measurement of anisotropy of FITC-Ova-BiP to Hsp70 or C70, the exitation maximum was set to 492 nm and the emission maximum to 519 nm, as determined by exitation- and emission scans. Slits

were set to 10 (exitation) and 20 (emission). For determination of binding constants, fluorescent peptide was incubated with increasing amounts of Hsp70 or C70 and with or without nucleotide for 30 min before end-point anisotropy readings were taken. Care was taken that r had reached stable values. For time-dependent binding studies, Hsp70 or C70 was incubated with fluorescent peptide and nucleotide at 25 °C directly in the cuvette, with continuous anisotropy readings taking place every 8 sec, until the reactions reached saturation.

III.4 Cell biological methods

III.4.1 Culturing of mammalian cells

Unless stated otherwise, murine cell lines were cultured in very low endotoxin (VLE)-RPMI 1640 containing stable glutamine, supplemented with 10 % fetal calf serum (FCS) and antibiotics (penicillin/streptomycin solution, Biochrom KG) at constant temperature, humidity and atmosphere (37 °C, 5 % CO_2 and 95 % air). B3Z cells were additionally supplemented with 50 µM 2-mercaptoethanol (Invitrogen).

Human cell lines were cultured in DMEM supplemented with 10 % FCS, penicillin/streptomycin and 2 mM L-glutamine.

Cells growing in suspension were subcultured through addition of fresh, pre-warmed medium as required (at least three times a week) and always on the day prior to an experiment.

For subculturing of adherent cells, medium was removed and the cell layer was rinsed once with PBS followed by a five minute incubation with trypsin/EDTA

solution. The cell suspension was then diluted as required through addition of fresh medium.

III.4.2 Determination of cell number

Live cells were counted in a haemocytometer (Neubauer chamber) using trypan blue as a dye.

III.4.3 Binding assay with fluorescently labeled Hsp70 and N70

For analysis of binding behavior of Hsp70 and N70, cultured cells were washed twice with PBS after removal of culture medium. Adherent cells were washed off the culture flask without trypsin to preserve proteinaceous surface structures. $5*10^5$ – $2*10^6$ cells were incubated with 300 nM fluorescently labeled protein which had or had not previously been loaded with peptide and ADP. Surface binding of Hsp70-488 and N70-488 was performed for 45 min on ice (in PBS) to prevent unspecific binding and uptake events. Cells were washed three times in PBS containing 1 % FCS and cross-linked with 2 % PFA/PBS, before being subjected to FACS analysis.

III.4.4 Competition experiments

Raw 264.7 macrophages were incubated with 1 µM labeled Hsp70 (ADP) in the presence of a 10-fold excess of unlabeled Hsp70, N70 or C70 in the presence of BSA for 30 min on ice. Cells were then washed three times with TBS, fixed with 3 % PFA and analyzed by confocal microscopy.

III.4.5 Antibody staining

For flow cytometry analysis, cultured cells were detached without the use of trypsin and washed once with PBS, followed by 30-45 min incubation with the primary antibodies. Cells were washed twice before application of secondary antibodies (when necessary), followed by an additional 30-45 min on ice and in the dark. After 3 washes with ice-cold PBS, cells were resuspended in 500 µl 2 % PFA/PBS and subjected to flow cytometry.

For fluorescence microscopy, cells were grown on cover slips to about 50 % confluency. Cells were incubated with labeled proteins and peptides for 30 min on ice, washed three times with TBS and fixed with 3 % PFA for 20 min. Prior to application, Hsp70 and its domains were loaded with 100µM ADP for 30 min at 37 °C. Samples were embedded in Mowiol and stored in the dark.

III.4.6 Flow cytometry analysis

Fixed or unfixed cell suspensions at a concentration of $1*10^6$/ml – $5*10^6$/ml were analyzed in a FACSCalibur™ (BD Biosciences) with the Cellquest software. Live populations were gated as determined by size and light scattering properties in an FCS/SSC plot. At least a total of 10 000 events in the live cell gate were analyzed for surface fluorescence. Alexa-488 and FITC dyes were detected in with the machine's FL-1 filter, while red fluorescent dyes such as PE and Alexa 595 were detected in FL2. Background fluorescence was measured in the absence of labeled protein.

III.4.7 Transient transfection of mammalian cells

Transient transfections of cultured mammalian cells were performed through electroporation in Gene Pulser Xcell device (Biorad, Munich, Germany). Detached cells were washed once with cold medium and $1*10^6$ – $10*10^6$ cells per transfection were resuspended in 400 µl DMEM supplemented with 25 % FCS. Cell suspensions were mixed gently with plasmid DNA (typically 10 µg per transfection) and transferred to pre-chilled 4 mm gap electroporation cuvettes. Cells were pulsed with the following settings:

Table 6: Electroporation settings

Cell line	Setting	Capacity	Voltage	Time constant
HEK 293T	exponential	950 µF	240 V	variable
Raw 264.7	time constant	variable	280 V	30 ms

After electroporation, cells were allowed to recover at room temperature for 5 – 20 min, washed once with medium, and plated.

III.4.8 Lipopolysaccharid (LPS) treatment

For LPS treatment, macrophages were grown in medium supplemented with 1 µg/ml, 10 µg/ml or 20 µg/ml LPS in medium over night. The endotoxin was then removed by multiple PBS washes.

III.4.9 Cryopreservation and revitalisation

For storage of cell lines in liquid nitrogen, cells were detached without the use of trypsin, pelleted and resuspended in 10 % DMSO/FCS. After transfer into

cryopreservation vials, cells were frozen stepwise for 1 h at -20 °C, followed by 12 – 26 h at -80 °C and final transfer to liquid N_2.

Revitalisation of frozen cells was achieved through rapid warming of the cells at 37 °C. Cells were then washed once with warm medium and plated. Medium was exchanged on the following day in order to remove residual DMSO.

III.4.10 Cross-presentation assays

Cross-presentation assays were carried out in V-bottom 96 well plates. $5*10^4$ dendritic cells or macrophages were incubated together with the antigen of interest and $5*10^4$ B3Z cells in final volumes of 50 µl – 200 µl per well. All experiments were performed at least in duplicates, usually in triplicates. After 4 h at 37 °C, plates were centrifuged (450 x g) and supernatant was discarded. The pellets were washed once with PBS and resuspended in 180 µl freshly prepared Buffer Z supplemented with CPRG substrate per well. Sufficient lysis of the cells was achieved mechanically by 20 fold pipetting of the suspension.

Plates were covered with self-sticking transparent foil and transferred into a pre-heated Synergy HT plate reader (Bio-Tek, Bad Friedrichshall, Germany). The enzymatic reaction was allowed to proceed at 37 °C for 4 hours, with kinetic readings taking place every 15 min. Absorbance was measured at 570 nm and 650 nm, and the difference between the two values was determined (delta OD). Enzymatic activity of beta-galactosidase usually reached saturation after 1-2 hours. For data evaluation, the delta OD of wells containing no antigen was set as 1.

IV RESULTS

IV.1 Interaction of Hsp70 with the surface of APCs and non-APCs

Previous experiments performed in our and other laboratories had shown a necessity for receptor mediated endocytosis (RME) in cross-presentation of Hsp70-chaperoned peptide. RME has been proposed as the reason for the extremely efficient immune response generated by such complexes *in vivo*, and a set of receptors such as CD40 and CD91 have been suggested to act as uptake receptors for Hsp70.

To further characterize the interaction of Hsp70 with the surface of mammalian cells, recombinant human Hsp70 was purified and labeled fluorescently with Alexa-488, with a labeling efficiency of 2 molecules dye per molecule Hsp70. To ensure that labeling did not interfere with the molecular properties of Hsp70, ATPase assays were performed using labeled and unlabeled recombinant Hsp70 as well as Hsp70/Hsc70 isolated from pig brain. Labeled and un-labeled protein was shown to be active and the activity was comparable to that of purified porcine protein (data not shown).

To visualize surface binding of the chaperone to mammalian cells, labeled Hsp70, in its ATP-, substrate-free form as well as in its substrate-bound, ADP-form, was incubated with a set of cultured mammalian cells of different origin. Cells were incubated in the presence of labeled Hsp70 at low temperature (on ice) to inhibit nonspecific endocytosis. Surface binding was visualized by FACS analysis or fluorescent microscopy after removal of unbound material.

IV.1.1 Conformation-dependent binding of Hsp70 to the surface of APCs

ANA-1 murine macrophages, which have been previously shown to interact with the Hsp70 molecule, bound Hsp70 specifically (Figure 7A). Cos-7 cells, on the other hand, showed little or no cell surface staining through the Hsp70 molecule (Figure 7B), which implicates the participation of specific, cell-type dependent receptors in the binding event. While macrophages are professional antigen-presenting cells, Cos-7 cells are simian kidney cells which are not considered to have any immune-stimulating or cross-presenting activity. Similar results were obtained with Raw 264.7 macrophages and CHO chinese hamster ovary cells as demonstrated by fluorescent microscopy (Figure 7C and D). Confocal analysis of Hsp70 binding to mammalian cells was performed in collaboration with Sandra Zitzler at the ZBH in Heidelberg, Germany.

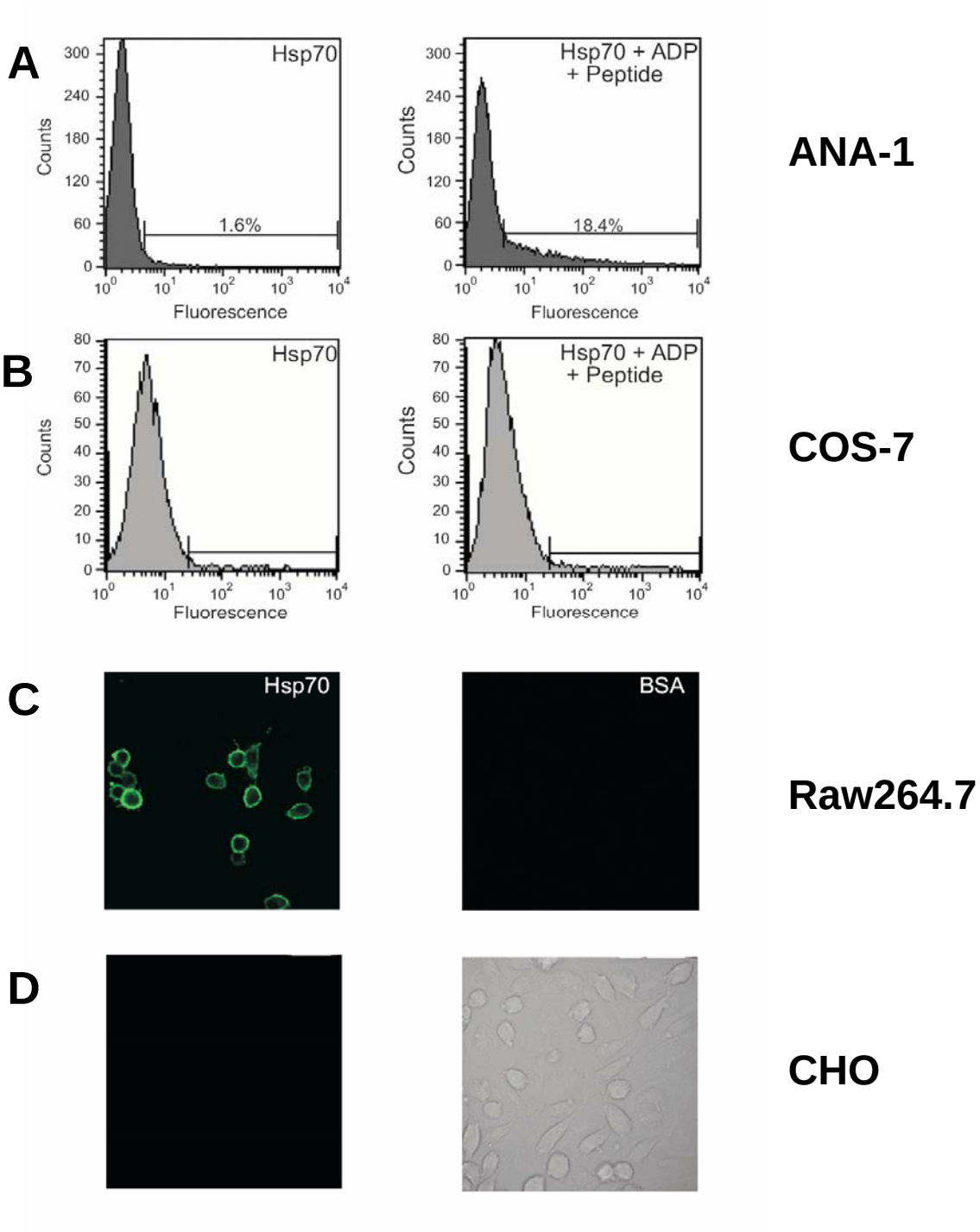

Figure 7: Binding of labeled Hsp70 to the surface of cultured mammalian cells.

(A) ANA-1 murine macrophages bind Hsp70 in its peptide-loaded, ADP-state but not in it's empty, ATP state. Intensity of surface fluorescence (x-axis) is plotted against cell counts. **(B)** Cos-7 (simian kidney cells) do not show cell surface labeling through Hsp70, regardless of its nucleotide state. **(C)** Raw 264.7 murine macrophages show clear surface staining with Alexa-488 labeled Hsp70 but not with labeled BSA. **(D)** CHO (chinese hamster ovary) cells do not bind Hsp70.

Interestingly, there was a marked difference between the binding behaviors of the two nucleotide states of Hsp70. While little or no binding to the macrophage surface could be shown for the open conformation of the molecule, a population of cells showed clear interaction with substrate-loaded Hsp70 in its ADP-bound state (Figure 7A). As is known from structural studies, Hsp70 undergoes a marked change in its conformation in dependence on its nucleotide- and substrate state, with the ADP-state representing tight substrate binding. Experiments had shown that chaperones are released *in vivo* during necrosis such as occurs in cancer and viral infection, but not through regulated apoptotic processes.

Such necrotic cell death is characterized by ATP-loss in the cytosol, which would lead to stable binding of substrate by Hsp70 during cell lysis and stabilize the HSP-peptide complex. The preferable recognition of the substrate-bound state of the chaperone by uptake receptors on antigen presenting cells could be a highly efficient mechanism to discriminate between empty and loaded Hsp70 molecules, preventing uptake of bulk material and leading to endocytosis only of cross-presentation competent, loaded chaperone molecules.

IV.1.2 Contribution of the Hsp70 SBD and NBD to the cell surface interaction

To better understand the mechanism of specific, conformation-dependent binding of Hsp70 to cells of the immune system, it was necessary to explore which functional domain of the molecule promotes this interaction. As previous results had shown a clear dependence on nucleotide (and substrate-) state of Hsp70 for surface binding, the first question to be addressed was whether the nucleotide binding domain alone of Hsp70 could specifically interact with the surface of cultured mammalian cells.

The ATPase domain of human heat shock protein 70 was purified from *E. coli* BL21 as a soluble His-tagged protein through affinity chromatography and ATP-agarose and labeled with Alexa 488 as described for Hsp70. ATPase activity of labeled protein was tested and found to be comparable to that of the unlabeled nucleotide-binding domain of the protein.

As with the full-length protein, binding assays were performed to analyze specificity of N-domain interaction with different cell types. Again, a clear dependence on the nucleotide-state of the protein was observed (Figure 8). These results led to the initial assumption that it is the N-terminal ATPase domain of Hsp70 that is responsible for specific binding of the molecule to the surface of immune cells. However, it is important to note that the binding pattern of N70 seems to be a different one than that of the full-length molecule. While incubation of macrophages with labeled Hsp70 leads to a smooth, homogenous labeling of the cells surface (Figure 7C), a more punctuate staining is observed with the amino terminal domain (Figure 8B).

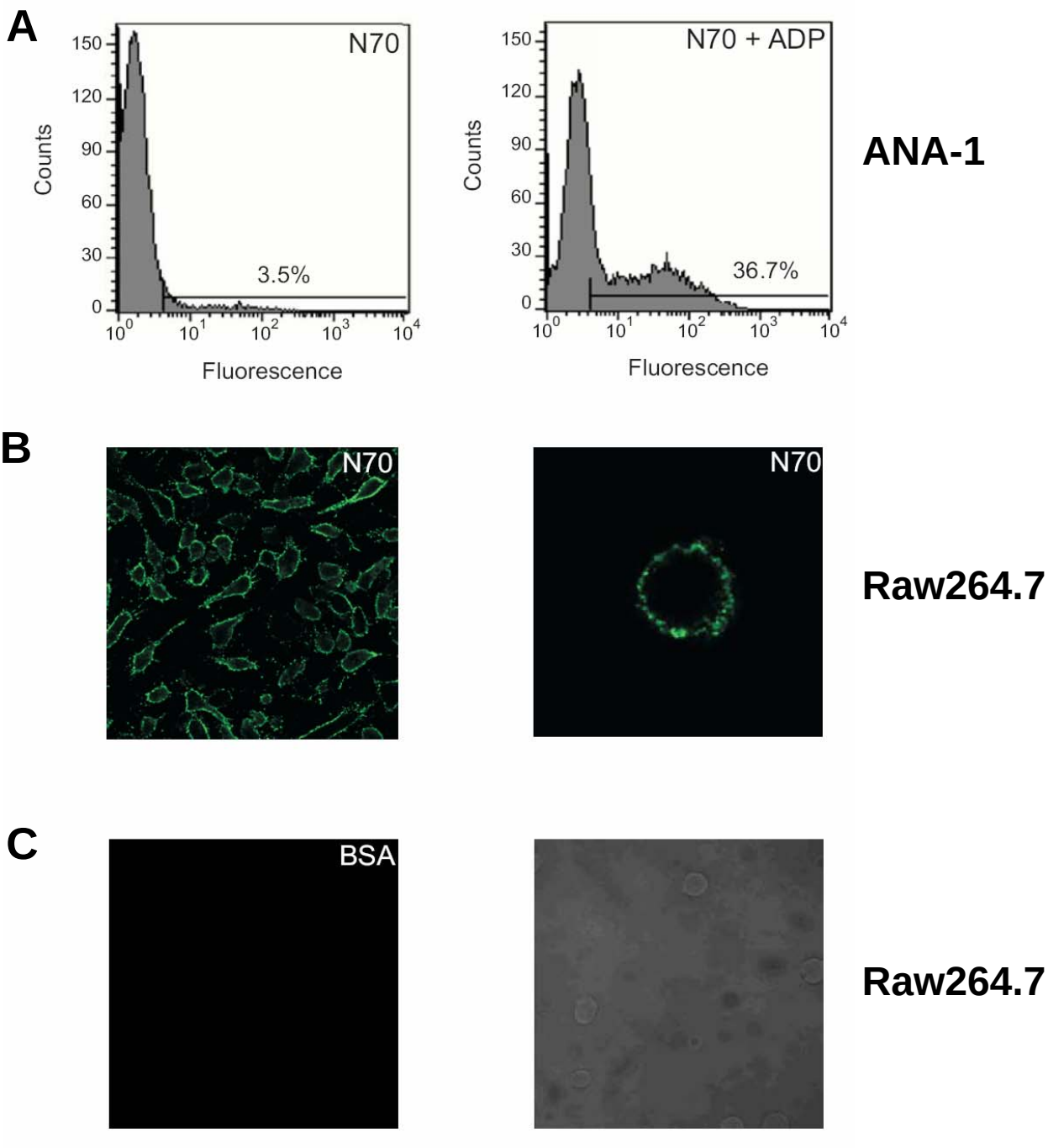

Figure 8: Interaction of the Hsp70 amino-terminal ATPase domain with ANA-1 and Raw 264.7 macrophages.

(A) ANA-1 macrophages bind Alexa-488 labeled N70 in a nucleotide-dependent manner. **(B)** Surface staining of Raw 264.7 macrophages with Alexa-488 labeled N70. **(C)** Alexa-488 labeled BSA shows no binding to the macrophage surface.

The different labeling patterns observed for the full-length molecule and the amino-terminal ATPase domain indicated that additional regions of the Hsp70 molecule may also play a role in the interaction with cell surface receptors. In order to further characterize this phenomenon, the C-terminal substrate binding domain (C70) of Hsp70 was purified and labeled with Alexa-633. C70 showed clear binding to the surface of antigen presenting cells, with a punctuate binding pattern similar to that of the ATPase domain (Figure 9). The binding of the SBD as well as the NBD of the Hsp70 molecule to the surface of the same cells could explain the differences in the observed binding patterns. If different receptors are responsible for the binding of each domain, resulting in punctuate binding pattern, a combination of both N- and C-terminus as supplied by the full-length molecule would result in the observed homogenous staining.

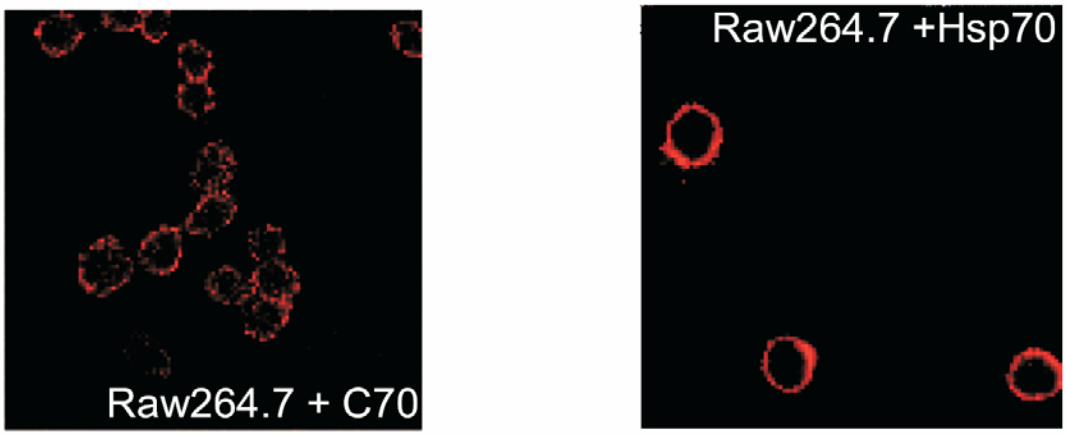

Figure 9: Binding of the substrate binding domain of Hsp70, C70, to the surface of macrophages.

The C-terminal substrate binding domain interacts with the surface of Raw264.7 macrophages, resulting in a punctuate staining pattern similar to that of N70 and in contrast to the homogenous staining generated by the full-length molecule labeled with the same dye.

Competition experiments were performed to investigate the binding properties of the respective domains. For this purpose, Raw 264.7 macrophages were incubated with 1µM fluorescently labeled Hsp70 or a mixture of labeled Hsp70 and unlabeled competitor for 30 min on ice. Cells were then washed and subjected to fluorescence microscopy. As expected for a specific, receptor-mediated interaction, the binding of labeled Hsp70 to Raw 264.7 macrophages could be fully competed with a 20x molar excess of the unlabeled molecule (Figure 10B).

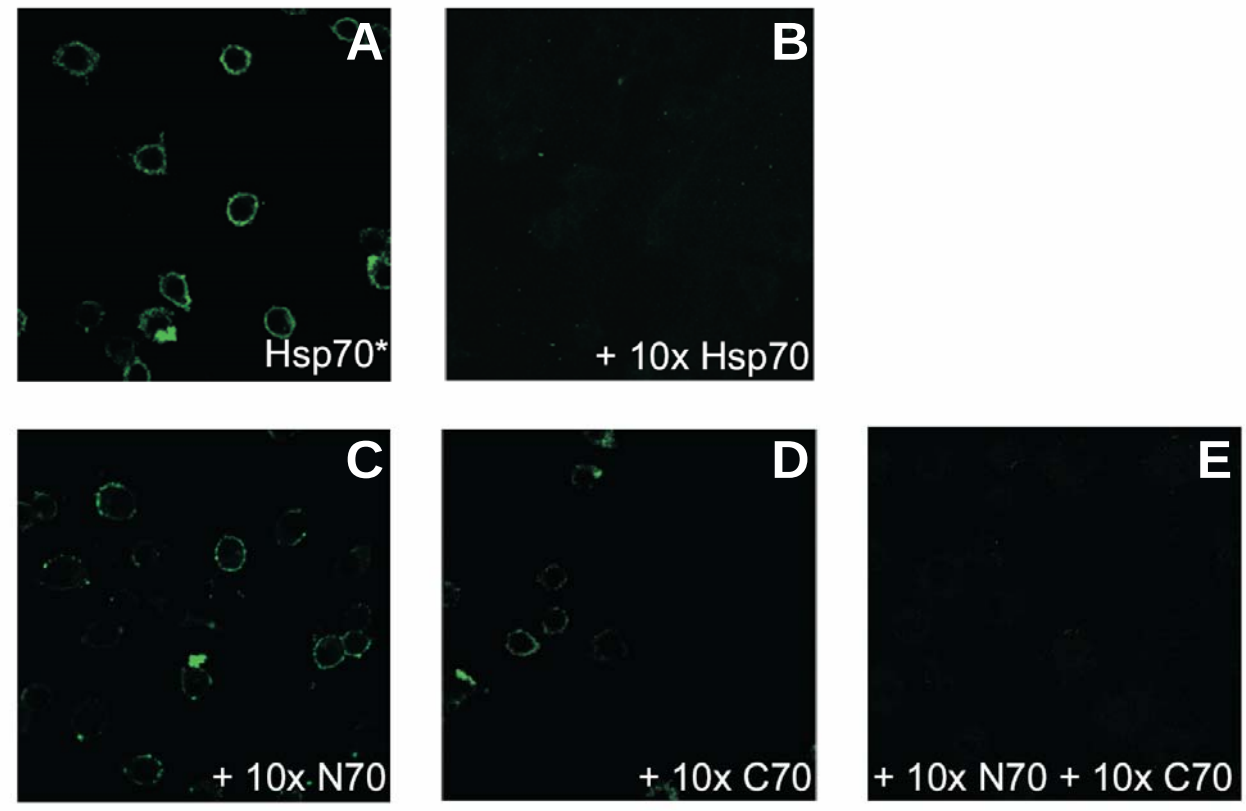

Figure 10: Only the combined application of both nucleotide- and substrate-binding domain can fully compete the binding of labeled Hsp70 to Raw264.7 cells.

(A) Surface staining of Raw264.7 macrophages through fluorescently labeled Hsp70. **(B)** A 10-fold molar excess of unlabeled Hsp70 is sufficient to fully compete the binding of labeled Hsp70. In contrast to the full-length molecule, the individual domains are not able to fully outcompete Hsp70-binding **(C)** and **(D)**, while the combination of both domains restores the competition **(E)**.

Importantly, an equally high concentration of the isolated NBD or SBD, respectively, could not fully compete the interaction with the full length molecule (Figure 10C and D), while a combination of both domains led to a complete loss of staining by the labeled full-length molecule (Figure 10E). All competition experiments were performed in the presence of a 10-fold excess of BSA to ensure specificity.

These competition experiments reinforced the hypothesis that both domains of the Hsp70 molecule play a role in cell surface interaction, leaving the question open which domain is necessary (and sufficient) to promote cross-presentation of the chaperoned peptide.

IV.1.3 Role of the proposed receptors CD40 and CD91 in the interaction of Hsp70 with the surface of mammalian cells.

As previously mentioned, several receptors have been implied in the interaction of Hsp70 with the surface of mammalian cells. CD40, a well-known signaling receptor, has been proposed as an uptake receptor for Hsp70, and the interaction between the two molecules has been mapped to the amino-terminus of the chaperone (Becker et al., 2002). In order to re-examine this interaction, ANA-1 macrophages, which have been shown to bind Hsp70 in a nucleotide dependent manner, were treated with lipopolysaccharide (LPS), which increases cell-surface expression of CD40, and analyzed for their ability to bind fluorescently labeled Hsp70. As expected, LPS-treatment resulted in an increase of CD40 expression by ANA-1 cells. Surprisingly, cell-surface interaction of the Hsp70 molecule was found to decrease after LPS-treatment of the macrophages, and disappeared completely after application of a higher dose of the endotoxin (Figure 11).

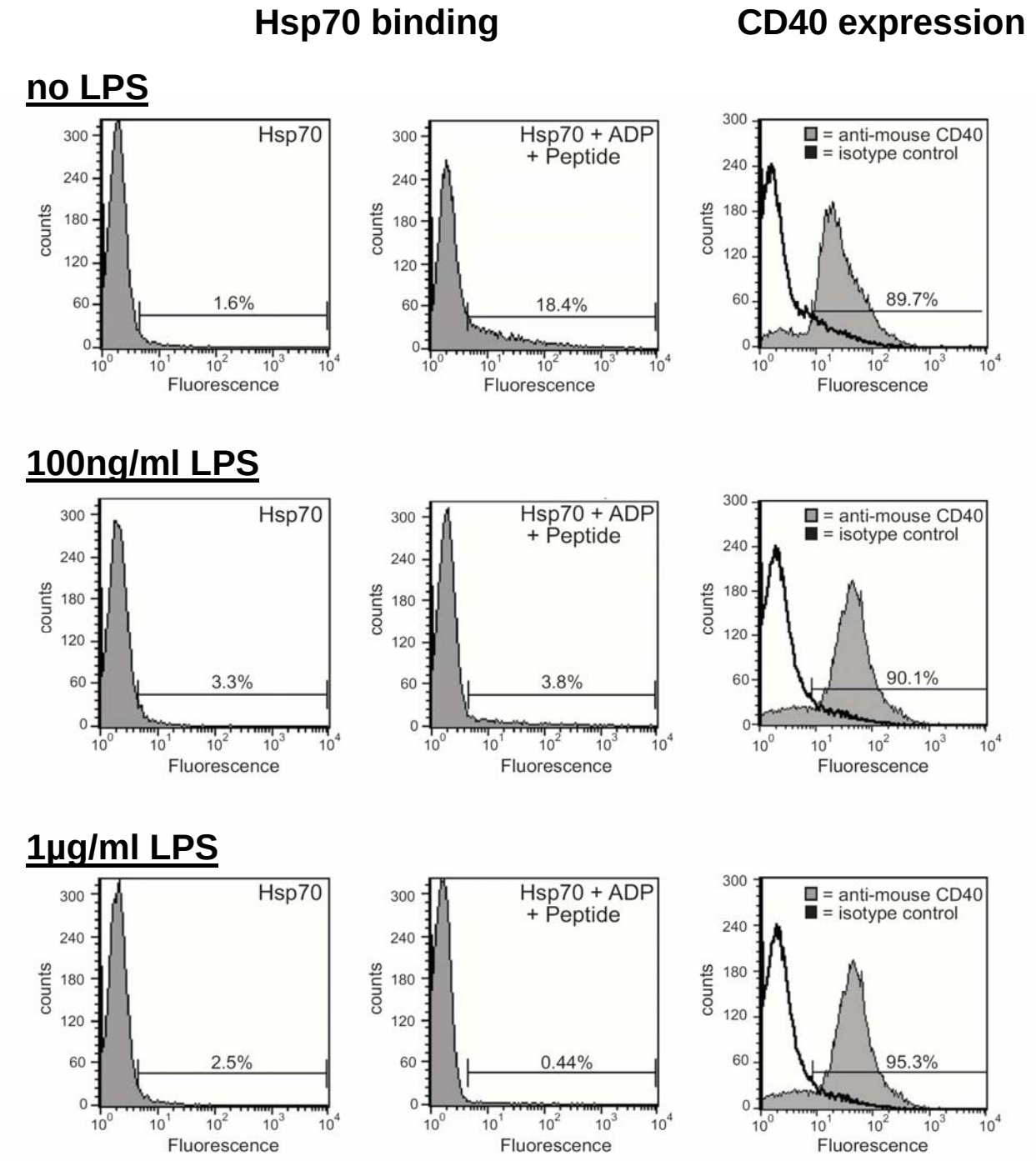

Figure 11: LPS treatment of macrophages results in increased CD40 expression but loss of Hsp70 binding.

ANA-1 macrophages were treated with indicated amounts of LPS over night before performing Hsp70 binding studies. While the expression of CD40 on the macrophage surface was increased due to endotoxin application, the binding of labeled Hsp70 exhibited a concentration-dependent decrease after treatment of cells with LPS.

Since treatment of macrophages with LPS has additional effects other than a rise in CD40 expression, a second method was chosen to confirm the findings that Hsp70-surface interaction is not dependent on CD40 as the sole receptor. HEK 293T cells, which are capable of expressing very high levels of protein when transfected with the appropriate vector, were chosen as a model system. Electroporation of the fibroblasts with a human CD40 construct resulted in very high surface expression of the molecule, as measured by the application of PE-labeled antibodies against hCD40 and subsequent FACS analysis (Figure 12). Since the interaction of CD40 with Hsp70 had been mapped to the amino-terminal domain of the molecule, transfected cells were analyzed for their capability to bind labeled N70 in a nucleotide-dependent manner.

As depicted in Figure 12A, the transfection of HEK 293T cells with the human CD40 construct was highly efficient, with virtually the entire population expressing the receptor. Nevertheless, the specific and nucleotide-dependent binding of the Hsp70 amino-terminal domain did not increase upon expression of human CD40 on the cell surface. Taken together, it remains debatable whether CD40 has a relevant role in the interaction of Hsp70 with the surface of mammalian cells. It can not be excluded, however, that CD40 acts as a component of a multi-receptor complex, and an additional co-receptor would be necessary to promote functional interaction with Hsp70.

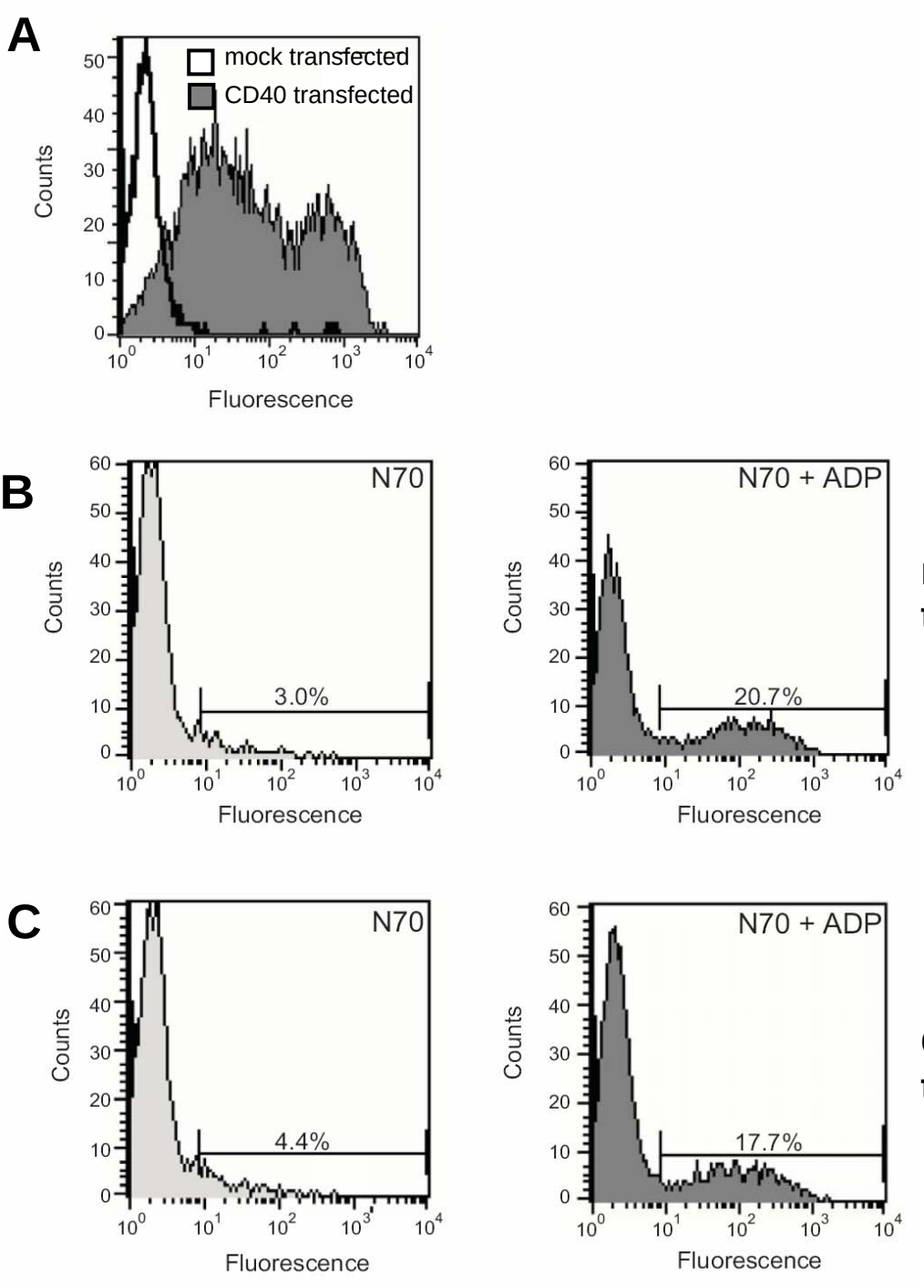

Figure 12: Over-expression of CD40 has no influence on the binding of fluorescently labeled N70 to the surface of HEK293T cells.

(A) High CD40 expression on the surface of transfected cells. Nucleotide-dependent binding of labeled ATPase domain to CD40 transfected **(C)** and mock transfected **(B)** cells is not influenced by over-expression of the receptor.

In addition to CD40, CD91 has been proposed as a receptor not only for Hsp70, but also for gp96 and other chaperones, as well as multiple other ligands (Basu et al., 2001). The interaction between Hsp70 and CD91 was re-examined in the lab of Felix Wieland. The group compared the two macrophage lines Raw 264.7 and Raw 309 Cr.1 as well as CHO cells stably transfected with CD91 (CHO+CD91).

As shown for Ana-1 macrophages in this study, Raw 264.7 macrophages lost their capability to bind Hsp70 after LPS-treatment. Western blot analysis of cell membranes showed unaltered expression of CD91 on the macrophage surface, challenging the connection between Hsp70 binding and CD91 expression.

Raw 309 Cr.1 macrophages, on the other hand, had been described by Basu et al. as a cell line that does not express CD91 and, accordingly, does not bind Hsp70 or cross-present Hsp70-chaperoned peptide. In contrast to this perception, Felix Wieland's group could show that the cells indeed did bind labeled Hsp70, and this binding was further increased through LPS treatment. Expression of CD91, on the other hand, was severely reduced after LPS treatment. Our group performed experiments clearly showing that the macrophages were capable of cross-presenting peptide chaperoned by Hsp70, and this property was not diminished after LPS treatment (data not shown).

Taken together, these results clearly argue against CD91 as a receptor for Hsp70, as neither binding nor cross-presentation properties are affected by its presence or absence.

IV.2 Cross-presentation of peptide chaperoned by Hsp70

The highly effective immunization properties of Hsp70 rely on its ability to bind peptides and shuttle them to antigen presenting cells, resulting in receptor-mediated uptake and subsequent cross-presentation of these peptides or fragments derived from them on MHC class molecules of the APCs.

In order to investigate the cross-presenting capabilities of Hsp70, an *in-vitro* system was established which allows loading of the chaperone with peptide and following the processing and presentation of the peptide by cultured antigen presenting cells. The model peptide chosen for this purpose, Ova-BiP, consists of a high affinity ligand for the peptide-binding site of Hsp70 (BiP) and a dominant T-cell epitope (Ova), connected by a glycine-serine-glycine linker. The peptide (Figure 13A), originally described by Moroi and collegues (Moroi et al., 2000), was shown to induce cellular immunity *in vitro* and rejection of tumors *in vivo* when applied in complex with Hsp70. The primed CTL responses in mice were more potent than in those immunized with epitope (complexed to Hsp70) alone, supporting the concept that peptide must be bound to the chaperone in order to be efficiently cross-presented. Hsp70/Ova-BiP complex formation *in vitro* in the presence of ATP is saturable, with a calculated dissociation constant of 332nM for FITC-labeled peptide, and can be further stabilized through addition of a molar excess of ADP (Figure 13B).

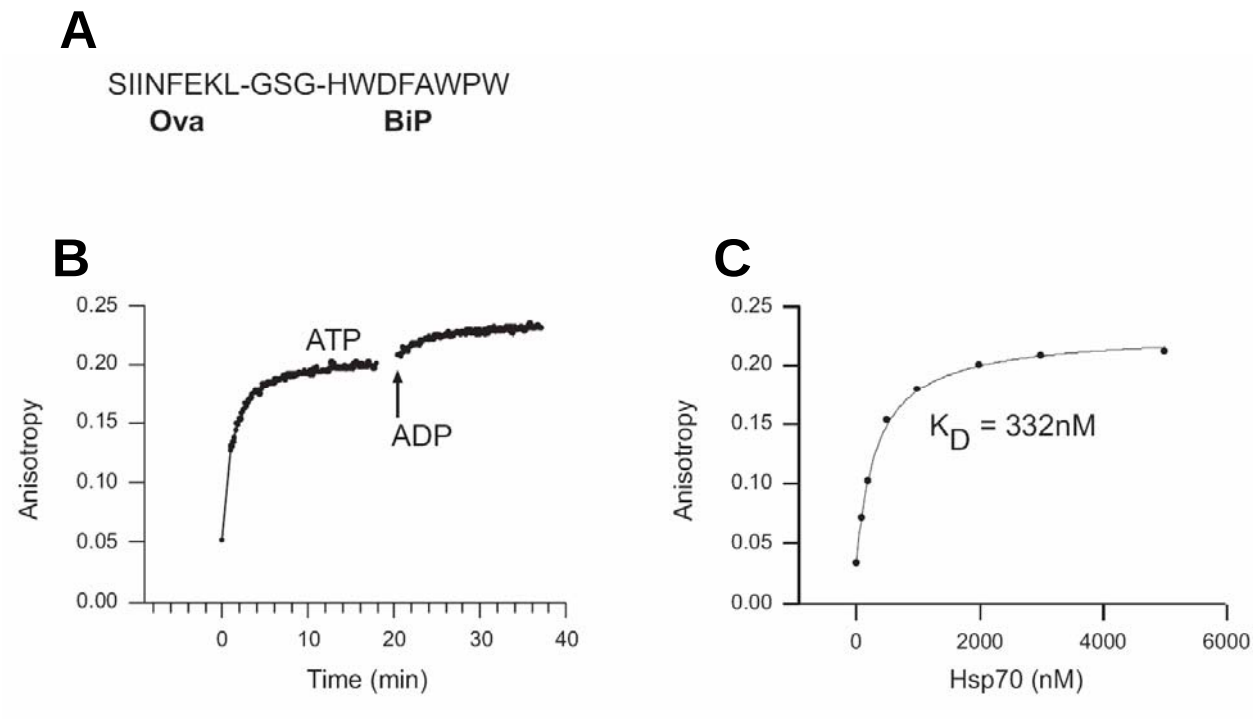

Figure 13: Ova-Bip is an in-vitro substrate of Hsp70

(A) Sequence of Ova-BiP: SIINFEKL is an ovalbumin-derived epitope for the murine MHC class I H-2k(b). (B) *In vitro* loading of Hsp70 with hybrid peptide: FITC labeled Ova-BiP was incubated at room temperature for the indicated time in the presence of 100 µM ATP. Addition of 1 mM ADP further increases and stabilizes the substrate-loaded state of Hsp70. **(C)** Binding curve of FITC-Ova-BiP to Hsp70.

The generated Hsp70-peptide complexes can be separated from free peptide by gel filtration and administered to cultured dendritic cells or macrophages. The cells, capable of cross-presentation, take up the complexes and present, after processing the hybrid peptide, the SIINFEKL antigen on their MHC class I K(b) molecules. This cross-presentation can then be monitored by two methods. The murine antibody 25-D1.16 specifically recognizes the SIINFEKL epitope when presented on H2-k(b) molecules. This antibody was purified from hybridoma supernatant and fluorescently labeled with Alexa 555, allowing detection of cross-presented model peptide by fluorescence microscopy or FACS.

For more efficient and better quantifiable detection of cross-presented antigen, the B3Z cell line was employed. Originally described by the group of Nilabh Shastri (Karttunen et al., 1992), B3Z is a Ovalbumin/k(b) specific T-cell hybridoma which is activated upon encounter of cells presenting SIINFEKL on their H2-k(b) molecules. Highly sensitive detection of T-cell activation is possible through a simple colorimetric assay. This assay makes use of a stably transfected lacZ reporter construct under the control of the nuclear factor of activated T cells (NF-AT) element of the human interleukin 2 (IL-2) enhancer. Due to this construct, occupancy of the TCR triggers not only the secretion of IL-2 but also the production and intracellular accumulation of lacZ, which can then be visualized by lysis of the T cells and their incubation with chromogenic substrates.

IV.2.1 Peptide chaperoned by Hsp70 is cross-presented efficiently by dendritic and macrophage cell lines

Using the described approach, it was possible to monitor the cross-presentation of Hsp70-chaperoned peptides by antigen-presenting cells. DC2.4, a murine dendritic cell line, and Raw 309, a murine macrophage cell line, were chosen for this purpose since they express the appropriate MHC class I haplotype and are professional antigen presenting cells capable of cross-presentation. As shown in Figure 14, Ova-BiP peptide, when complexed to Hsp70, was efficiently cross-presented by the antigen-presenting cells at concentrations which are not sufficient for free peptide to be presented. The amount of Hsp70 molecules carrying peptide after separation from free peptide was estimated as 20%, as determined by the group of Felix Wieland (Stefan Hancke, personal communication) and in agreement with previously published data

(Moroi et al., 2000). Hsp70-peptide complexes applied to the APCs did not contain free peptide (Figure 14B). Dendritic cells cross-presented Hsp70-bound peptide more efficiently than macrophages, which is not surprising given that DCs are thought of as the principle cross-presenting cells *in vivo*.

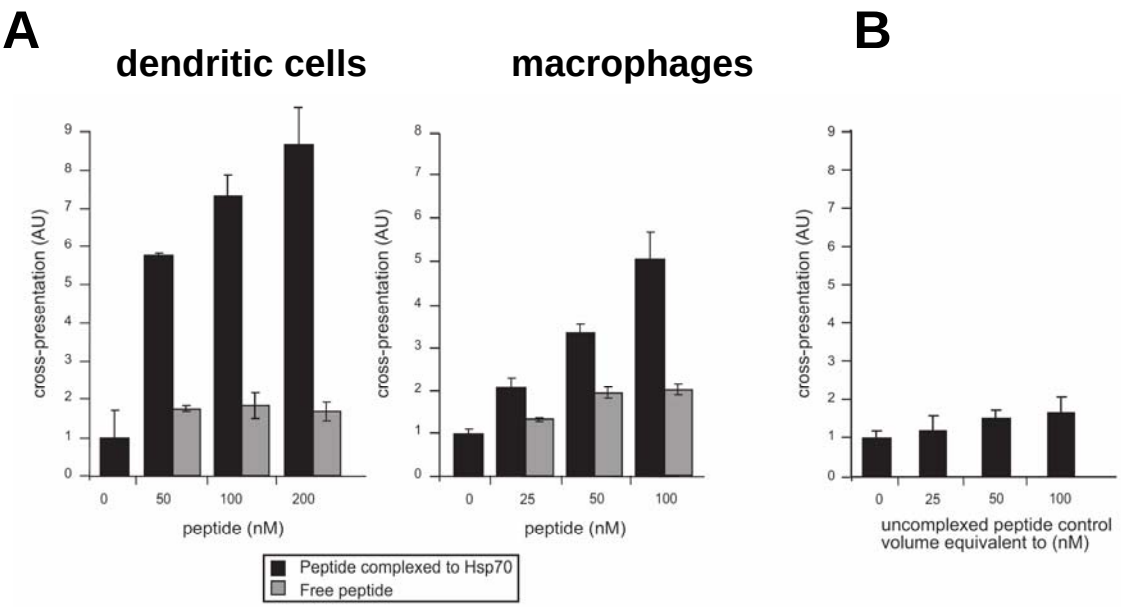

Figure 14: Cross-presentation of Hsp70-chaperoned Ova-BiP by DC2.4 and Raw309

(A) Incubation of dendritic cells or macrophages with peptide complexed to Hsp70 results in its processing and cross-presentation, whereas free peptide is not efficiently cross-presented at respective concentrations. **(B)** Fractions corresponding to those applied in A but without Hsp70 were tested for cross-presentable material.

IV.2.2 Receptor mediated endocytosis is responsible for uptake and cross-presentation of Hsp70-chaperoned peptide

The described cross-presentation of Hsp70-chaperoned Ova-BiP is efficient enough to be certain that it is due to receptor-mediated endocytosis. Additionally, it has been made sure that there is no free peptide present in the Hsp70/Ova-BiP preparations. However, in theory, peptide alone is also competent to be cross-presented by APCs if its concentration is high enough, and Hsp70/Ova-BiP could additionally be taken up by unspecific mechanisms. In order to completely rule out the participation of any kind of unspecific uptake of "bulk material", an assay was developed that allows the initial association of Hsp70/Ova-BiP to the surface of the antigen presenting cell on ice, where no endocytotic activity can take place. Unbound material is then washed away and cells are warmed to physiologic temperature where they can take up receptor-bound material. Under these conditions, there is no cross-presentation of protein/peptide taken up by unspecific mechanisms such as macropinocytosis, and only Hsp70-chaperoned peptide is cross-presented (Figure 15).

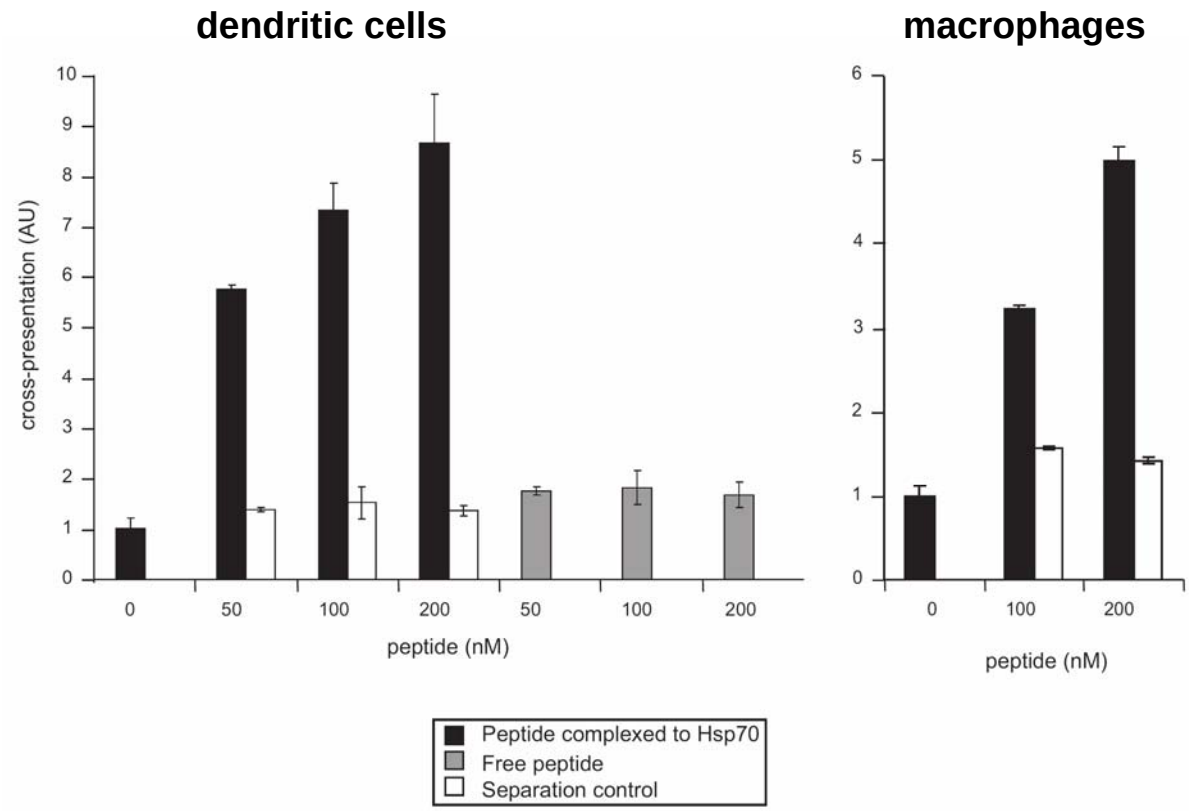

Figure 15: Uptake and cross-presentation of Hsp70/Ova-BiP after association on ice.

DC2.4 dendritic cells or Raw309 macrophages cells were incubated with indicated amounts of peptide, either complexed to Hsp70 or free in solution for 20 min on ice, followed by extensive washing before warming to 37 °C and incubation with B3Z T-cell hybrids. Hsp70-chaperoned peptide was efficiently cross-presented while no cross-presentation above background was detectable for administered free peptide.

These results provide a direct link between the previously shown specific binding of Hsp70 to the surface of antigen presenting cells and the capability of Hsp70-peptide complexes to elicit potent immune responses against the peptide chaperoned by them. Not only does Hsp70 “chaperone” the peptide to antigen presenting cells, but its binding to specific receptors on the surface of these cells leads to uptake, processing and cross-presentation of the peptides at concentrations not sufficient for free peptide to be cross-presented. Given these results and in

combination with the previous data, which showed a clear interaction of Hsp70 with the surface of antigen presenting cells both through its ATPase- and substrate-binding domain, it was know possible to address the question which of the determined interactions is the one responsible for the cross-presenting capabilities of the heat shock protein.

IV.2.3 Analysis of the domains of Hsp70 necessary for cross-presentation

In order to analyze the cross-presenting capabilities of the individual Hsp70 domains, two separate strategies were chosen. As the N-terminal domain showed nucleotide-dependent binding to the surface of antigen presenting cells, it was first addressed as a candidate for mediating the interaction responsible for the cross-presenting potential of Hsp70. Since purified N70 lacks the substrate binding domain and thus cannot be loaded with peptide, an alternative strategy was chosen. Previously published data had shown successful immunization of mice with fusions between Hsp70 and substrate protein (Suzue et al., 1997), albeit the chaperone used for these studies was not of mammalian but of mycobacterial origin and is therefore only remotely related to the *in vivo* situation. Members of the same group later mapped the minimal portion of the Hsp70 molecule necessary to generate a CTL response against the polypeptide fused to it to the amino acids 161-370, which are located in the amino terminal ATPase domain (Huang et al., 2000). However, since one half of the ATP-binding domain cannot be sufficient to retain chaperone activity, the authors conclude that the ability of their fusion proteins to elicit $CD8^+$ T cells does not depend on the HSP moieties' chaperone properties.

In order to examine whether an interaction of such fusion proteins with the immune system takes place on the cellular level, similar fusions between murine Hsp70 or murine N70 (aa 161-370) and an ovalbumin domain containing the SIINFEKL epitope (OVA) were cloned and purified from *E.coli*. The fusion proteins were then incubated with DC2.4 and B3Z cells as described for the Hsp70/Ova-BiP complexes. While application of the Hsp70-OVA fusion resulted in some cross-presentation of the SIINFEKL peptide, albeit considerably weaker than achieved through generated Hsp70/Ova-BiP complexes, the N70-OVA fusion did not exhibit any cross-presenting capability at all (Figure 16).

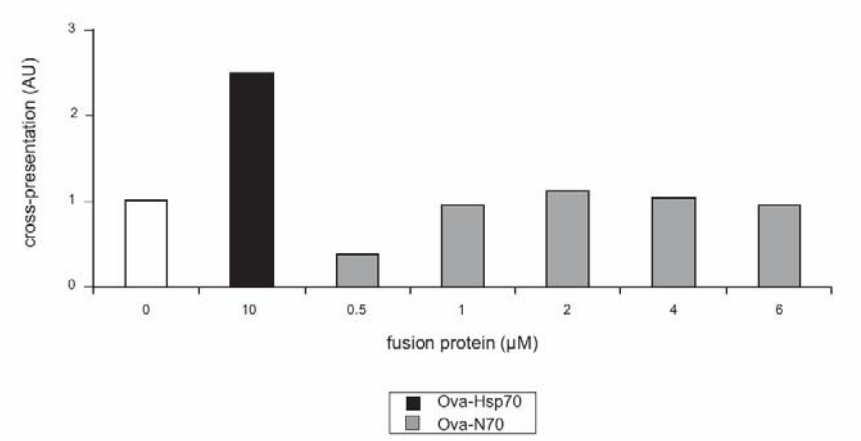

Figure 16: Cross-presentation of SIINFEKL derived from fusion proteins.

DC2.4. cells were capable of cross-presenting SIINFEKL derived from the Hsp70-OVA fusion at high concentrations, while no cross-presentation is detectable from the N70-Ova fusion.

These results indicate that the N-terminal domain alone is not responsible for the high efficiency of cross-presentation conferred by the full-length Hsp70 molecule. In order to analyze whether the C-terminal, substrate binding domain (SBD) might be sufficient to cross-present chaperoned peptides, the SBD of human Hsp70 was purified

from *E.coli* as described for the full-length molecule. Isolated substrate binding domain (C70) bound Ova-BiP hybrid peptide *in vitro*, with binding kinetics that are much slower than those of the full-length molecule and a calculated K(d) of 730nM for FITC-labeled Ova-BiP (Figure 17a and b).

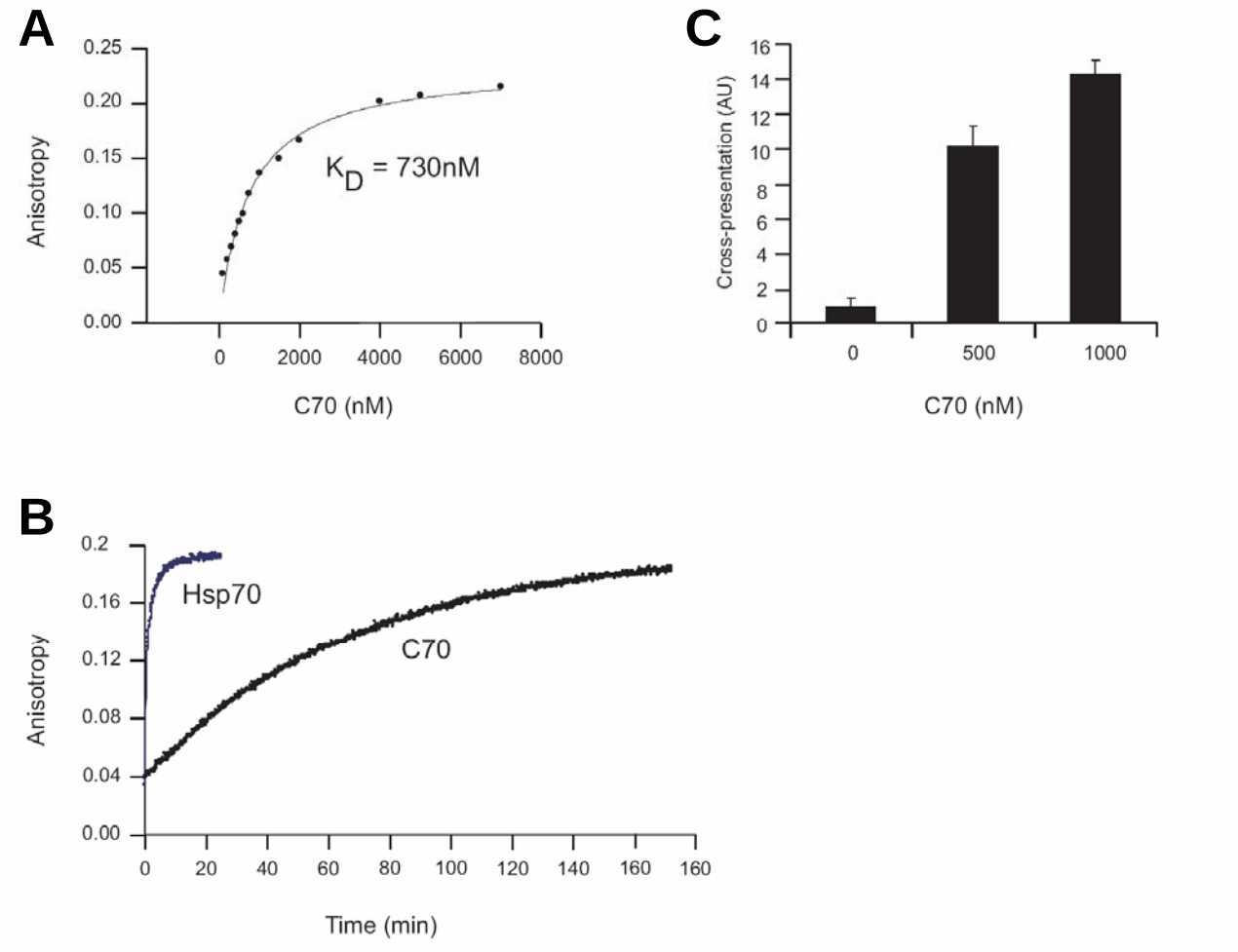

Figure 17: The substrate binding domain is competent of chaperoning complexed peptide to the cross-presentation pathway of dendritic cells.

(A) Binding curve of FITC-labeled Ova-BiP to C70 as measured by fluorescence anisotropy. **(B)** binding kinetics of C70 as compared with Hsp70. **(C)** highly efficient cross-presentation of C70-chaperoned peptide.

As shown in Figure 17C, the isolated substrate binding domain of Hsp70 efficiently chaperoned bound Ova-BiP to the cross-presentation pathway of dendritic cells. Given the roughly double dissociation constant of C70/Ova-BiP as compared to Hsp70/Ova-BiP, and taking into consideration that in the case of Hsp70, 20 % of the molecules are loaded with peptide, one could estimate that approximately 10 % of the C70 molecules employed for the cross-presentation studies are loaded with hybrid peptide. This would imply cross-presentation efficiencies of C70 which are roughly two-fold higher that those of the full-length Hsp70. This suggests strongly that the Hsp70 substrate binding domain is not only sufficient to bind and stabilize the peptide, but also to be the principal interaction partner of a cross-presentation-competent uptake receptor.

As for the full-length molecule, it was possible to discriminate between a possible situation in which C70 merely binds and stabilizes Ova-BiP and a specific, RME-induced cross-presentation process by experimentally separating the binding of C70/Ova-BiP complexes to the surface of dendritic cells from the uptake and processing steps.

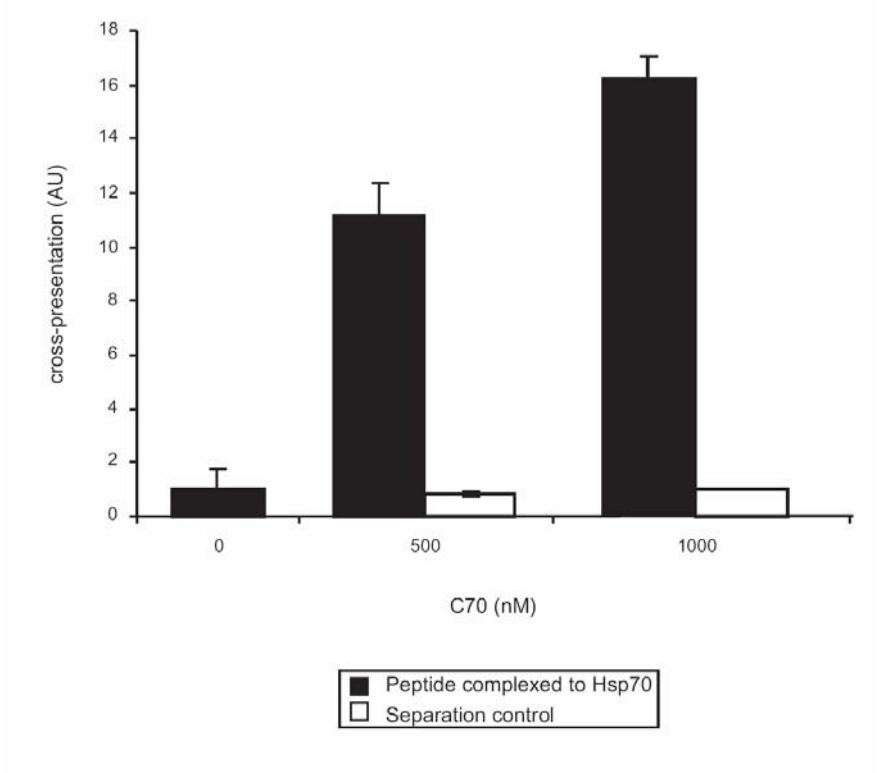

Figure 18: Uptake and cross-presentation of C70/Ova-BiP after association on ice.

In such an experimental setup, the carboxy-terminal substrate binding domain of Hsp70 mediated highly efficient cross-presentation of the chaperoned peptide (Figure 18). This data provides a direct link between the observed cell-surface interaction of the SBD with the chaperone's cross-presentation capability and clearly proves that this domain alone is necessary and sufficient for the molecules cross-presenting capability.

IV.3 Analysis of cellular compartments involved in uptake of the Hsp70/peptide complex

In order to obtain a more complete picture of the cross-presentation pathway taken by peptide-loaded Hsp70, an attempt was made to identify the cellular compartments involved in uptake and processing of the complex. For this purpose, an experimental system was developed which allows selective down-regulation of individual components of the cellular endocytic machinery. Dominant-negative mutants of four different small GTPases (DynI, Rab5, Rab7, and ARF6) as well as some constitutively active variants were utilized to specifically interfere with key steps of the endocytic process. Since the transfection efficiencies of dendritic cells or macrophages are too low to achieve a clear effect on the cross-presentation capabilities of a total cell population, a co-transfection approach was chosen to make sure that only cells expressing the respective mutant protein are being evaluated. For this purpose, Raw 264.7 macrophages, which were previously shown in this study to specifically interact with Hsp70/Ova-BiP complexes but lack the H-2K(b) molecule necessary for detection of cross-presentation by the B3Z system, were transiently transfected with an expression vector for k(b), thus gaining the ability to cross-present SIINFEKL peptide derived from Hsp70/Ova-BiP to B3Z cells (Figure 19A). For down-regulation of respective small GTPases, the macrophages were co-transfected with a mixture of k(b)-encoding and mutant-encoding plasmids in a ratio of 1:3, thus statistically ensuring that only such cells express the H-2k(b) molecule which also express the dominant negative mutant. Since the B3Z system as well as the 25D1.16 antibody solely recognize cells cross-presenting SIINFEKL on surface k(b) molecules, virtually

the entire analyzed cell population in this experimental setup carries the co-transfected plasmid.

Dynamin I (as well as Dynamin II) is a marker of early endocytotic steps where it is mainly involved in the pinching off of clathrin-coated pits. Many receptor-mediated uptake processes are known to be dependent on this molecule, although some lipid-raft mediated pathways as well as macropinocytosis and phagocytosis have been shown to be independent of Dynamin. The dominant negative Dyn I K44A mutant is defective in its GTPase cycle and is known to cause a total inhibition of clathrin dynamics, while permitting receptor-mediated endocytosis by other means ((Boll et al., 2004; Damke et al., 1994).

In order to correctly interpret the effect of Dyn I K44A expression in Raw 264.7 macrophages on the cross-presentation of Hsp70 chaperoned peptide, it was crucial to determine whether the dominant negative Dynamin mutant has an impact on the expression level of the k(b) molecule itself. Since the available antibody against H2-k(b) is not sensitive enough to register changes in the expression level of the molecules, an approach was chosen which allows the employment of the highly sensitive B3Z system for this purpose. It is known that empty MHC molecules on the cell surface can be stabilized and become receptive for exogenously administered SIINFEKL peptide through cross-linking with paraformaldehyde (Rock et al., 1992). K(b) molecules loaded in such a manner can then be directly quantified by measuring B3Z response. In this as well as all following experiments, quantification of loaded K(b) molecules was performed by measuring B3Z response rather than employing the 25D1.15 antibody, as the antibody staining was not sensitive enough to register changes in expression/cross-presentation levels.

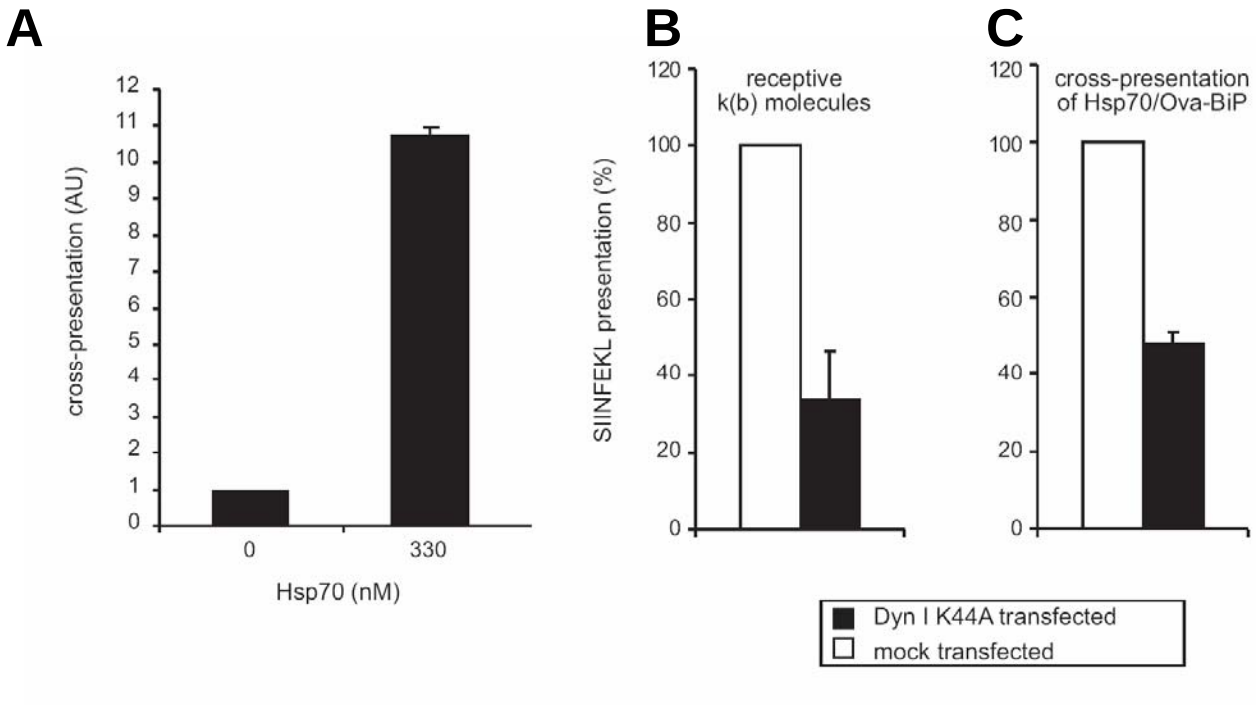

Figure 19: Effect of DynIK44A on cross-presentation of Hsp70/Ova-BiP

24h post transfection, Raw 264.7 macrophages were harvested and analyzed for K(b) expression and cross-presentation of Hsp70/Ova-BiP. **(A)** Macrophages cross-present SIINFEKL derived from Hsp70/Ova-BiP to B3Z when transfected with H-2k(b). **(B)** Raw 264.7 co-transfected with K(b) and Dyn I K44A express only ~30 % of the MHC class I molecules as compared with mock transfected. **(C)** Cross-presentation of Hsp70/Ova-BiP by Dyn I K44A transfected cells is reduced to a similar amount as the level of k(b) molecules.

The expression of the dominant negative Dynamin I mutant resulted in significant decrease of H-2k(b) surface molecules (Figure 19B). Such an effect can possibly be explained by the fact that fluid phase uptake is known to increase upon RME inhibition. This down-regulation of MHC class I molecules to 34 % must be considered when quantifying the effect of the dominant negative mutant on Hsp70 mediated cross-presentation.

To access the role of clathrin-mediated endocytosis on the cross-presentation of Hsp70-chaperoned peptides, co-transfected macrophages were incubated with Hsp70/peptide complexes before quantifying cross-presentation as described in materials and methods. The cross-presentation of Hsp70-bound Ova-BiP was determined to be 48 % (data from three independent experiments) as compared with the cross-presentation of the complex by cells transfected with empty vector. Taking into account that the quantification of k(b) molecules had revealed a decrease of MHC class I molecules (34 % as compared to mock-transfected) on the surface of co-transfected cells, it can be assumed that the observed reduction of cross-presentation is due to the lack of surface k(b) molecules and not to an interference in cross-presentation pathways. This would imply that Dynamin I does not play a relevant role in the cross-presentation of Hsp70-chaperoned peptide. In agreement with this observation, microscopic analysis of the uptake pathway taken by Hsp70/Ova-BiP complexes revealed that the complexes are localized in transferrin-negative early endosomal structures shortly after uptake (data not shown).

The described approach was used to analyze the effects of other small GTPase mutants on MHC level and cross-presentation of Hsp70/Ova-BiP. Rab 5 was chosen as a marker of early endosomes and its mutants Rab5 S34N (dominant negative) and Rab Q79L (constitutively active) were co-transfected with H-2k(b) as described for Dyn I. Rab7 is a marker of late endosomes and lysosomes, and Rab7 Q67L is a dominant negative form of the protein.

As shown in Figure 20A, transfection of Raw 264.7 macrophages with the Rab 5 and Rab 7 mutants resulted in an extremely high fluctuation of k(b) expression under identical experimental conditions. In average, both Rab 5 mutants had little effect on MHC surface level, while the Rab 7 mutant resulted in elevated k(b) expression. This stabilization of MHC molecules could be due to inhibited lysosomal degradation. Accordingly, cross-presentation in the co-transfected cells was differentially affected by the mutant GTPases. While Rab 5 seems to result in a general decrease of Hsp70-mediated cross-presentation of Ova-BiP, Rab 7 left the cells relatively unaffected.

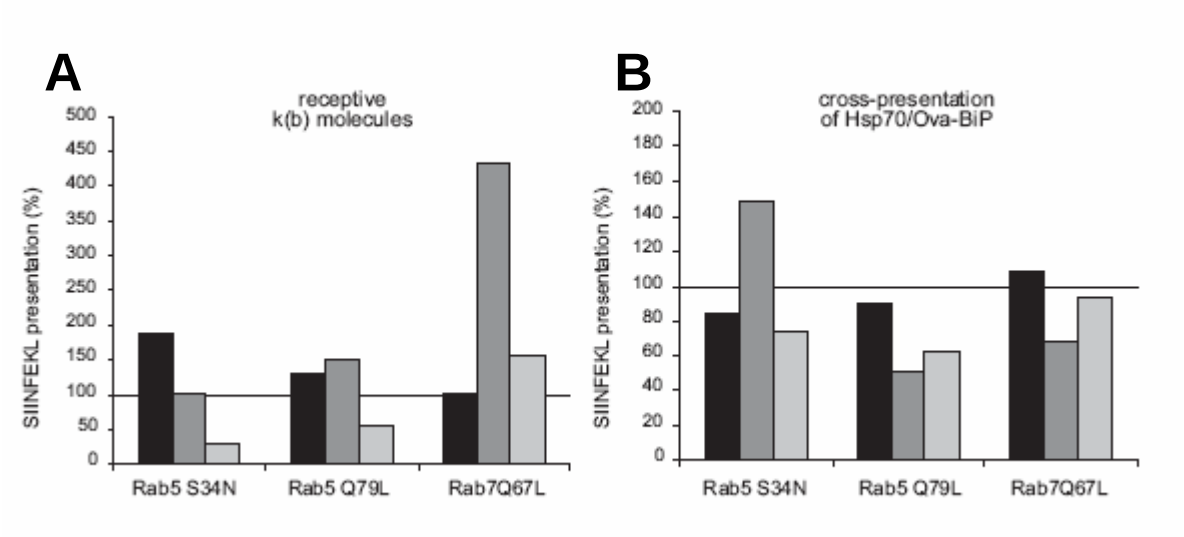

Figure 20: Effect of Rab5 and Rab7 mutants on MHC level and cross-presentation.
(A) k(b) levels vary dramatically in cells co-transfected with Rab5 or Rab7 mutants as compared with mock transfected. Grey shades represent individual experiments. **(B)** Cross-presentation of Hsp70/Ova-BiP cells transfected with Rab 5 and Rab 7 mutants.

Confocal studies with fluorescently labeled Hsp70 and Ova-BiP revealed a co-localization of the complex in a Rab5 positive compartment within minutes after uptake, while only unloaded Hsp70 could be detected in lysosomal structures (data not shown).

Initial experiments were also performed with mutant forms of the ADP ribosylation factor 6 (ARF 6). ARF6 is a small GTP-binding protein which plays a role in membrane recycling, phagocytosis, and cortical actin organization. It has been shown to participate in the recycling of MHC class I molecules, regulating a branch of dynamic tubular intermediates used by major histocompatibility class I molecules to traffic through vesicles between endosomes and the plasma membrane (Massol et al., 2005), and is thought to play an important role in targeting recycling endosomes to the plasma membrane (D'Souza-Schorey et al., 1998). It has also been shown to participate in macrophage phagocytosis, as T27N, a dominant negative form of the ADP ribosylation factor 6, has been shown to inhibit phagocytosis via receptors for the Fc portion of immunoglobulins (FcR) (Niedergang et al., 2003).

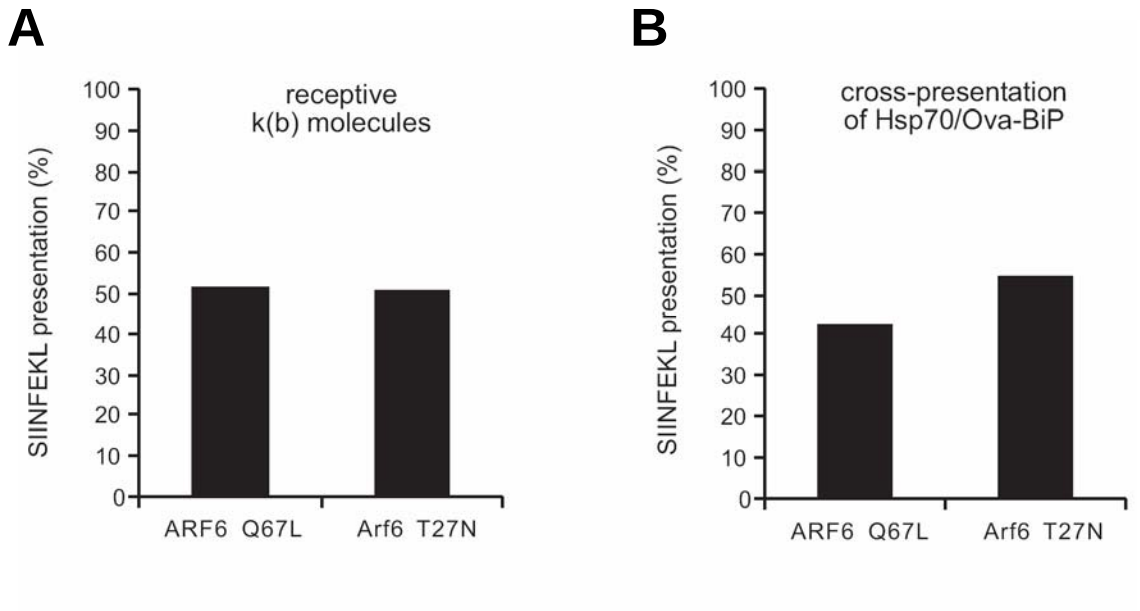

Figure 21: Effect of ARF6 mutants on MHC level and cross-presentation.
(A) k(b) levels are reduced to 50 % in macrophages transfected with ARF6 mutants as compared to mock transfected cells. **(B)** Cross-presentation of Hsp70/Ova-BiP cells transfected with dominant negative ARF6 mutants.

As expected, transfection of Raw 264.7 macrophages with the dominant negative mutants ARF6 Q67L and ARF6 T27N resulted in a strong decrease of co-expressed k(b) surface level (Figure 21A). Cross-presentation of Hsp70-bound Ova-BiP was not affected stronger than k(b) levels (Figure 21B), suggesting a cross-presentation pathway independent of ARF6. Further experiments need to be performed to exclude a role of ARF6, and with it recycling endosomes, in Hsp70-mediated cross-presentation, but the data collected until now indicates a clathrin-independent receptor-mediated endocytosis of Hsp70-peptide complexes, followed by a participation of Rab5 positive early endosomes. Thus it appears that a participation of late endosomes, lysosomes or recycling endosomes can possibly be ruled out.

V DISCUSSION

V.1 Interaction of Hsp70 with the surface of antigen presenting cells

Heat shock proteins have been described as potent tumor vaccines and are currently being deployed in clinical trials. They were identified as tumor-specific antigens, and mice immunized with HSPs purified from a tumor are protected from a subsequent challenge with the tumor of origin. The specificity of the immune response triggered by HSPs was ascribed to the peptides bound by them. As a direct consequence of the role of heat shock proteins as chaperones, they are thought to associate with a wide spectrum of peptides that reflects the entire antigenic repertoire of a cell.

Hsp70-peptide complexes have been shown to be released from cells dying from a number of pathological conditions, and the terminal stages of necrosis may also favor stable antigen binding by Hsp70, as intracellular ATP levels decline and peptides become locked onto the ADP-associated Hsp70 molecule during necrotic cell death (Calderwood et al., 2005b).

The first event in the interaction of extracellular Hsp70 with the immune system is the binding to the surface of professional antigen presenting cells (pAPCs). This binding event was characterized in the current study. For this purpose, recombinant human Hsp70 as well as its amino-terminal ATPase domain (N70) and carboxy-terminal substrate binding domain (C70) were purified and fluorescently labeled, with an emphasis on retaining functionality. A variety of cell lines were tested for their ability to bind Hsp70 and N70. It could be shown that the binding event is

specific for cells of the immune system (macrophages and dendritic cells), while non-immune cells such as COS-7 and CHO did not show surface staining. This selective binding clearly supports the concept that APCs express one or more receptor molecules on their surface which are not expressed by other cells.

Further analysis of the binding behavior of Hsp70 to APCs revealed that the association of the chaperone with cell membranes is dependent on the molecule's conformation. Hsp70 interacts with the putative receptors only in its substrate-bound, ADP-form, while the open conformation shows little or no surface staining. This phenomenon excludes the possibility that the interaction between Hsp70 and its receptor(s) is mediated by the chaperone-properties of the molecule, in a setting in which the receptor(s) would mimic a substrate of Hsp70 and the chaperone would bind this substrate in its peptide binding pocket. Physiologically, a preference of Hsp70-receptors for the closed conformation of the molecule would ensure uptake only of substrate-loaded chaperone and not empty, "useless" molecules, thus enhancing the specificity of the immune response. Accordingly, immunization with *in-vitro* generated or purified HSP-peptide-complexes is known to be effective only when peptide is carried by the chaperone and not when the two are administered separately. On the molecular level, it is plausible that a receptor is able to distinguish between the two forms of Hsp70, as the molecule experiences a considerable conformational change upon substrate binding and ATP hydrolysis.

A number of cell surface molecules have been proposed as receptors for Hsp70, the most prominent ones being CD91 and CD40. The function of these molecules as

Hsp70 receptors was critically evaluated in the present study. In the case of CD40, ANA-1 macrophages were treated with lipopolysaccharide (LPS) and analyzed for Hsp70 binding. While LPS treatment resulted in a significant increase in CD40 expression, binding of the chaperone was strongly reduced upon treatment with the endotoxin. This implies that while there is no causal connection between Hsp70 binding and CD40 expression, there is an effect of LPS stimulation on the expression of the Hsp70 receptor(s). LPS stimulation of macrophages ultimately results in the activation of gene transcription, leading to a wide variety of rearrangements in the cell. It can be assumed that the expression of Hsp70 receptors is also affected by these rearrangements. Additionally, seems plausible that the chaperone interacts only with non-activated antigen presenting cells, as it itself has been shown to provide an activation signal to the innate immune system.

As the previously described interaction between Hsp70 and CD40 had been mapped to the aminoterminal domain of the chaperone (Becker et al., 2002), binding experiments were also carried out with the isolated NBD. Human embryonic kidney cells (HEK), transfected with a highly expressed CD40 construct, showed no increase of N70 binding as compared with mock transfected cells. This further underlined the concept that CD40, if at all, is not the sole receptor for Hsp70 binding.

In the case of CD91, we were able to exclude this molecule as a receptor for Hsp70. Binding of Hsp70 to the surface of macrophages was entirely independent of CD91 expression, as a macrophage cell line which does not express the molecule (Raw 309) showed clear surface staining with Hsp70. The same cell line could also be shown to readily cross-present Hsp70-bound peptide, a property clearly dependent on

the presence of a receptor for Hsp70. Additionally, the group of Felix Wieland in Heidelberg could show that a cell line stably transfected with CD91 (CHO-CD91) did not show any surface binding of the chaperone. The CD91 molecule expressed by these cells was shown to be functional via the endotoxin A assay. In accordance, LPS-stimulated Raw 264.7 macrophages, like ANA-1 macrophages, lost their ability to bind Hsp70, while surface levels of CD91 showed no alteration.

CD91 has been described as a receptor not only for Hsp70, but also for the chaperones Hsp90, calreticulin and gp96 (Basu et al., 2001). It is a large transmembrane protein also known by the names of LRP (LDL-receptor related protein), α_2-macroglobulin-receptor and ApoE-receptor. It belongs to the family of scavenger receptors and as such binds a large variety of different substrates. It is feasible that a molecule with such a large variety of binding partners would also interact with Hsp70 under certain experimental conditions, but the specificity of interaction as described in this study could not be provided by such a promiscuous receptor.

Based on the observation that Hsp70 binds to the surface of antigen presenting cells in a conformation-dependent manner, the question was addressed which part(s) of the molecule contribute to these interactions. The aminoterminal ATPase domain displayed a binding behavior similar to that of the full-length molecule, demonstrating surface staining only in its ADP form. Interestingly, N70 displayed a punctuate binding pattern on the APC membrane, in contrast to the homogenous staining observed for the complete molecule. The substrate binding domain of Hsp70, C70, was also assayed for surface binding and revealed a punctuate pattern similar to that of

N70. This was the first indication that Hsp70 possibly possesses multiple interaction partners on the plasma membrane of APCs, and different moieties of the molecules interact with individual partners. This would be in agreement with the multiple functions that have been ascribed to it as an “immuno-chaperone”.

Further evidence for the existence of multiple cell-surface receptors for Hsp70 was gained by competition experiments. The individual domains were examined for their capability to compete with each other for binding of the macrophage membrane. As expected for receptor-mediated interactions, the binding of labeled full length Hsp70 molecules could be fully out-competed by addition of a 20-fold excess of unlabeled Hsp70. In a similar manner, unlabeled N70 competed with N70 and C70 competed with C70, verifying the specificity of interaction with cell-surface receptors. In contrast, the individual domains were not able to fully compete the binding of the full-length molecule, but rather reduced the homogenous staining to a punctuate pattern. Only a combination of both domains could fully interfere with the binding of Hsp70 to the macrophage membrane. Based on these observations, it becomes clear that at least two different receptors are responsible for binding of Hsp70 to the cell membrane, one of them interacting with the nucleotide binding domain and the other with the substrate binding domain of the chaperone.

Further analysis of the differential binding behavior of the individual domains was performed by our collaborators in the lab of Felix Wieland. N70 and C70 were labeled with different fluorescent dyes and incubated with Raw 264.7 macrophages. Confocal microscopy revealed heterogeneous staining with little overlap between the two domains, suggesting the binding of N70 and C70 to different micro-domains of the cell membrane.

This phenomenon was accessed biochemically through separation of macrophage membranes into raft and non-raft fractions after incubation of cells with biotinylated Hsp70, N70 or C70. The fractions were then separated by SDS-PAGE and subjected to western blot analysis. HRP-tagged Cholera toxin was used as a marker of lipid rafts, as it interacts specifically with the raft glycolipid GM 1. While Hsp70 and C70 was detectable both in raft and in non-raft fractions, N70 could only be found outside of lipid rafts, further underlining the concept of differential binding sites for the individual domains of Hsp70.

V.2 Cross-presentation of Hsp70-peptide complexes

The interaction of Hsp70 with antigen presenting cells results in stimulation of both innate and adaptive immune mechanisms. The activation of the adaptive immune system against peptides carried by Hsp70 is now understood to the extent that the complexes are taken up by receptor-mediated endocytosis (RME) and the peptides are processed (if necessary) and cross-presented on MHC class I molecules.

In this study, an *in vitro* system was established which allowed loading of Hsp70 with antigenic peptide and detecting the cross-presentation of the processed peptide in cultured dendritic cells and macrophages. Experiments performed with this system revealed that both dendritic cells and macrophages were capable of cross-presenting peptide chaperoned by Hsp70, and this cross-presentation was highly efficient at concentrations at which free peptide was not cross-presented.

The question was addressed whether a direct connection exists between the described cell surface interaction of Hsp70 and the cross-presentation of chaperoned peptide. An assay allowing uptake only of membrane-bound Hsp70-peptide complexes

was developed and revealed a clear correlation between the two processes. The binding of Hsp70 to its cell-surface receptors clearly results in the cross-presentation of peptide bound by it on the APCs' MHC class I molecules. Hsp70 thus does not merely act as a chaperone of peptides derived from dying cells, possibly protecting them from premature degradation in the blood stream, but indeed mediates the uptake of these peptides by cells capable of cross-presentation. We assumed that at least one of the interactions between the chaperone and the APC surface described in the previous section contributes to its cross-presentation capabilities. As the amino-terminal nucleotide binding domain cannot bind peptides, a fusion protein consisting of the NBD and a polypeptide containing antigenic peptide was employed. Incubation of antigen presenting cells with this fusion protein did not result in cross-presentation of the peptide, suggesting that the interaction between N70 and the APC surface is not the one responsible for Hsp70's cross-presenting competency.

The substrate-binding domain of Hsp70 was purified and shown to be competent for loading with antigenic peptide *in vitro*. Cross-presentation experiments performed with C70-peptide complexes revealed a pronounced ability of the SBD to chaperone peptides onto the cross-presentation pathway of antigen presenting cells. As for the full-length molecule, this cross-presentation ability could be linked to the described binding of the C-terminal domain to the surface of antigen presenting cells.

The conclusion that C70 alone is the principle cross-presenting domain of the Hsp70 molecule is a key step in the understanding of Hsp70-mediated cross-presentation. It could be hypothesized that the second interaction described in this study, that of N70 with differential microdomains on the APC membrane, results in the previously mentioned activation of innate immune mechanisms. Experiments

addressing this possibility are currently being carried out. Such a situation would allow a separation between the two main consequences of Hsp70 treatment, namely general immune stimulation and antigen-specific responses, thus facilitating exact dosage of each compound in clinical application. C70 is easy to purify and readily interacts with antigenic peptide, and as such could possibly be useful for immunization with complexes generated *in vitro*. The binding mechanism is separated from ATP binding and cleavage, which could possibly result in higher stability of chaperone-peptide complexes *in vivo*. It should become useful as an interaction partner for future screens which will be necessary to identify an uptake receptor for Hsp70/peptide complexes.

V.3 Uptake pathway of Hsp70/peptide complexes

In order to fully understand the mechanism by which Hsp70 chaperones peptides onto the cross-presentation pathway of antigen-presenting cells, it was necessary to investigate the pathway taken by the complexes within the cross-presenting cell.

Several independent models for antigen cross-presentation are currently being discussed, the general consensus being that the nature of the antigen defines which of the pathways is taken within the antigen presenting cell. Three main possibilities have been described for the fate of endocytosed antigens determined for cross-presentation: the “endosome-to-cytosol” pathway, the “recycling endosome” model as well as the “ER-phagosome model”.

In this study, two parallel approaches were chosen to gain understanding of the cellular compartments involved in Hsp70-mediated cross-presentation. Our laboratory employed dominant-negative mutants of small GTPases in order to specifically

interfere with endocytic processes and monitor the functional consequences of such interferences on the efficiency of cross-presentation. In parallel, our collaborators at the BZH in Heidelberg used fluorescently labeled Hsp70 and peptide to microscopically track the uptake of the complexes in cells capable of cross-presenting Hsp70-peptide complexes.

The dominant negative mutants employed in the study allow the interference with specific endocytic processes, in contrast to the frequently applied chemical inhibitors which have multiple, vaguely defined effects. A co-transfection approach was used which enabled the measurement of antigen cross-presentation with the B3Z cell line, allowing highly sensitive detection of MHC class I peptide presentation.

Unfortunately, the high sensitivity of the assay also resulted in a considerable fluctuation of the collected data, giving stable results only for one of the employed mutants, namely Dynamin I.

Interference with the formation of clathrin-coated pits by down-regulation of DynI activity resulted in a reduced cell-surface expression of MHC class I molecules. This can possibly be explained by the fact that inhibition of clathrin-coat formation initially leads to an increase of fluid phase uptake.

Hsp70-mediated cross-presentation, on the other hand, was unaffected by dynamin inhibition, supporting a clathrin-independent uptake pathway for the chaperone. Data from confocal microscopy confirmed this finding, showing an early localization of labeled Hsp70/Ova-BiP complexes in transferrin-negative compartments. Transferrin is a typical marker of clathrin-coated pits. Clathrin-independent uptake has been described for phagocytic mechanisms carried out by

macrophages and dendritic cells, usually participating in the endocytosis of pathogens or, in experimental setups, latex beads. The increasingly popular model of ER-mediated phagocytosis describes a direct fusion of ER- and plasma-membrane resulting in phagocytosis (Desjardins and Griffiths, 2003; Gagnon et al., 2002) and, in some cases, in cross-presentation of the internalized material (Guermonprez et al., 2003; Houde et al., 2003).

The next endocytic step taken by Hsp70-peptide complexes was investigated by analyzing the role of Rab 5 in their uptake and cross-presentation. Rab 5 is a marker of early endosomes and plays a central role in membrane fusion (Mohrmann and van der Sluijs, 1999). It has also been described to be localized in phagosomes (Desjardins et al., 1994) where it is thought to promote the interaction of endosomes and phagosomes (kiss and run) (Duclos et al., 2000).

Confocal microscopy revealed an early co-localization (after 2 min at 19 °C) of Hsp70/Ova-BiP with Rab-5 molecules, indicating an accumulation of the chaperone-peptide complexes in early endosomes or phagosomes, a process typical of receptor-mediated endocytosis. Accordingly, data from the transfection of macrophages with GTPase mutants indicated an inhibitory effect of Rab 5 interference on Hsp70-mediated cross-presentation.

The participation of ER structures in the cross-presentation pathway was examined through co-localization studies with PDI, an ER-resident enzyme. Interesstingly, an early localization of fully internalized Hsp70/Ova-BiP complexes in PDI-positive compartments could be shown. This could be a first indication of an

interaction of ER-structures with endosomal vesicles in the proximity of the plasma membrane.

Inhibition of Rab 7, a lysosomal marker, had no effect on Hsp70-mediated cross-presentation. When analyzed microscopically, a co-localization with Lyso Tracker could only be shown for empty, unloaded Hsp70 molecules, possibly indicating a degradation of the chaperone after "antigen-delivery" to the cross-presentation pathway of the antigen-presenting cell.

Similarly, preliminary experiments performed with mutated ARF6, a GTPase responsible for membrane fusion of recycling endosomes, showed no effect on the cross-presentation efficiency of Hsp-bound peptide, arguing against the so-called "recycling endosome" model.

A detailed understanding of the pathway of Hsp70-mediated cross-presentation will require further analysis. The employment of chemical inhibitors of endocytic processes would be one possibility to further underline the generated results. Additionally, APCs stably transfected with the available dominant negative mutants of small GTPases could possibly overcome the variability problem associated with transient transfection.

Based on the data at hand, a preliminary model can be proposed which resolves how Hsp70 delivers antigen to the cross-presentation pathway of APCs (Figure 22): Hsp70 is loaded with antigenic peptide in the cytosol of virally infected or tumor cells, from which it is released during necrotic cell death. A decrease of ATP concentration in the dying cell locks the chaperone in its ADP state and ensures tight substrate binding.

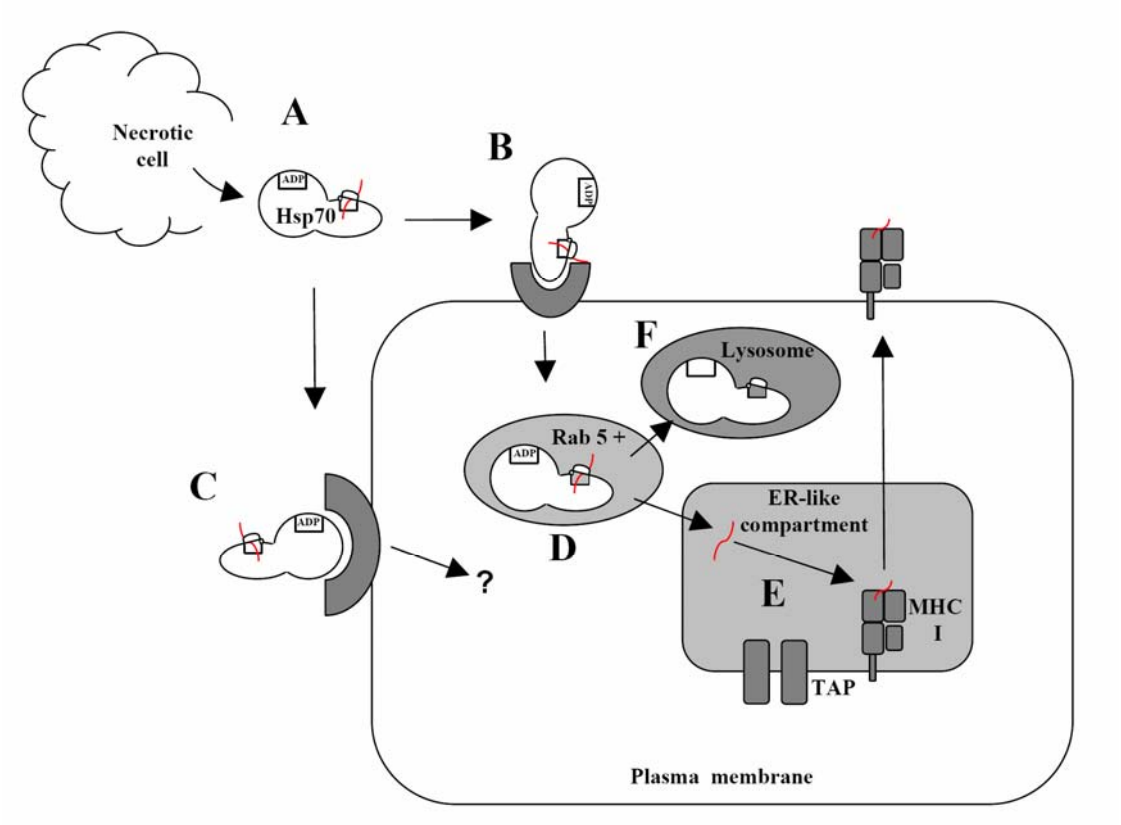

Figure 22: Model of Hsp70-mediated antigen cross-presentation

(A) Release of Hsp70/peptide complexes during necrotic cell death. **(B)** Binding of Hsp70-peptide complexes to uptake receptors on the APC surface is mediated by the chaperone's substrate binding domain. **(C)** Additionally, the nucleotide binding domain of Hsp70 can interact with the APC surface. **(D)** Internalized Hsp70/peptide complexes are transported into Rab 5 - positive endosomes, from which the antigenic peptides gain access to ER-like, cross-presentation-competent structures **(E)**, where they are loaded on MHC class I molecules and presented on the cell surface. **(F)** Empty Hsp70 molecules are transported into lysosomes where they become degraded.

Peptide-loaded Hsp70 specifically interacts with a set of receptors on the surface of antigen presenting cells. Uptake of the chaperone, resulting in cross-presentation of the delivered antigen, is mediated by the molecule's carboxy-terminal substrate binding domain. Other interactions, including that of the aminoterminal domain of Hsp70, are not yet fully understood, but might result in stimulation of the antigen presenting cell.

Receptor-mediated endocytosis of Hsp70/peptide complexes leads to a rapid uptake and accumulation in Rab 5 positive endosomal/phagosomal structures, from which the complexes gain access to MHC class I presentation competent, ER-like structures. While the bound peptide is loaded on MHC class I molecules and presented on the cell's surface, empty Hsp70 molecules are transported into lysosomes where they are degraded.

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VII APPENDIX

VII.1 Abbreviations

(p)APC	(professional) antigen presenting cell
aa	amino acid
ADP	adenosine 5'-diphosphate
Amp	ampicillin
APS	ammonium peroxodisulfate
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
C70	carboxy-terminal substrate binding domain
CTL	cytotoxic T-lymphocyte
CPRG	Chlorophenolred-β-D-galactopyroanoside
DC	dendritic cell
dn	dominant negative
DMSO	dimethyl-sulfoxide
Dyn I	Dynamin 1
E. coli	*Escherichia coli*
ER	endoplasmic reticulum
ERAD	ER associated degradation
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
HRP	horse radish peroxidase

HSP	heat shock protein
IPTG	isopropyl-β-D-1-thiogalactopyranoside
LPS	lipopolysaccharide
MΦ	macrophage
MHC	major histocompatability complex
N70	amino-terminal nucleotide binding domain of Hsp70
NAC	nascent chain associated complex
NBD	nucleotide binding domain
OD	optical density
PFA	paraformaldehyde
PBS	phosphate buffered saline
RAC	ribosome-associated complex
RME	Receptor mediated endocytosis
SBD	substrate binding domain
S. cerevisiae	*Saccharomyces cerevisiae*
TEMED	N,N,N'N-tetramethylethylendiamine
TF	trigger factor
TLC	thin layer chromatography

VII.2 Acknowledgments:

I thank H. Wagner for Raw, Cos-7, Ana-1, 293T and B3Z cell lines; K. Rock for the DC2.4 cell line; P. Ahmad-Nejad for human cDNA; H. Wagner and R. N. Germain for the 25D1.15 antibody and R.M. Vabulas for providing all of the above listed materials.

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