Heat Shock Proteins as Immunological Carriers

by

Kimiko Suzue

B.S., Biochemistry
University of Illinois, 1990

SUBMITTED TO THE DEPARTMENT OF BIOLOGY IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY
AT THE
MASSACHUSETTS INSTITUTE OF TECHNOLOGY

May 1998

© 1998 Kimiko Suzue
All rights reserved.

The author hereby grants to MIT permission to reproduce and to distribute publicly paper and electronic copies of this thesis document in whole or in part.

Signature of Author:_____
Department of Biology
May 1, 1998

Certified by:_____
Richard A. Young
Professor of Biology
Thesis Supervisor

Accepted by:_____
Frank Solomon
Chairman, Committee for Graduate Students

AUG 041998

Dedication

To my husband Noren, for all his love, for his unfailing support for my scientific career and for his contagious enthusiasm for research.

To my parents for their love and encouragement.

Heat Shock Proteins as Immunological Carriers

by

Kimiko Suzue

Submitted to the Department of Biology on May 1, 1998 in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biology at the Massachusetts Institute of Technology

ABSTRACT

The hsp70 protein functions as a molecular chaperone and is an integral component of the protein folding machinery in a cell. Interestingly, the *Mycobacterium tuberculosis* hsp70 protein has been found to be a major target of the immune response and is a dominant antigen for both B and T cells. We exploited the antigenic and chaperoning properties of mycobacterial hsp70 and used it as an adjuvant-free carrier protein to stimulate the immune response to an accompanying protein. We found that a recombinant HIV p24-hsp70 fusion protein could be administered in saline in the absence of adjuvant to elicit strong and long lasting immune responses to HIV-1 p24 . The anti-p24 IgG1 antibodies induced in p24-hsp70-immunized mice persisted at high levels for more than one year after immunization. Immunization of mice with p24-hsp70 elicited p24-specific Th1 and Th2 cells and thus splenocytes from the immunized mice exhibited p24 antigen-dependent proliferation and production of IL-2, IL-5 and IFN-γ.

The chaperoning properties of heat shock proteins prompted us to investigate whether an exogenously added soluble hsp70 fusion protein can prime antigen-specific MHC class I-restricted $CD8^+$ CTLs. Ovalbumin, when covalently linked to mycobacterial hsp70 and administered without adjuvant, was processed for presentation in the MHC class I pathway and elicited CTLs

in mice. In contrast, when administered as a mixture with hsp70, soluble ovalbumin failed to induce CTLs. The immune responses engendered by hsp70-ovalbumin fusion proteins can protect mice against a lethal challenge with ovalbumin-expressing tumor cells. These results imply that hsp70 fusion proteins have features that permit them to enter into cellular compartments that lead to MHC class I antigen presentation.

The effectiveness of hsp70 in eliciting an antigen-specific CTL response and a protective tumor response highly accentuates the utility of hsp70 in inducing immune responses to any antigen of interest. An hsp70 fusion protein can be utilized to induce immune responses against multiple B or T cell epitopes in the accompanying antigen of interest. Hsp70 fusion proteins may a practical, inexpensive strategy for inducing immunity to pathogens in man.

Thesis Supervisor: Richard A. Young
Title: Professor of Biology

Table of Contents

Section I

Introduction

Section I: Introduction

The purpose of this introductory section is to discuss the chaperoning functions and the immunogenicity of heat shock proteins. These aspects of hsp70 prompted me to study the *Mycobacterium tuberculosis* hsp70 protein as an 'immunological carrier'. I have taken advantage of these special properties of hsp70 and in the subsequent sections of this thesis, I will describe my work describing the exceptional manner in which hsp70 proteins are able to elicit both humoral and cellular immune responses against a covalently attached antigen of interest. Chapter 1 describes the structure and function of molecular chaperones and chapter 2 illustrates the poweful antigenic nature of heat shock proteins. The information contained in this section has been published as a review article review article: Suzue, K. and Young, R.A., Heat shock proteins as immunological carriers and vaccines. in: Stress-Inducible Cellular Responses (U. Feige, R. I. Morimoto, I. Yahara, B. S. Polla, eds.), Birkhauser/Springer, 77: 451-465 (1996).

Section I

Chapter 1. Heat shock proteins as molecular chaperones

Classic *in vitro* experiments by Anfinsen with ribonuclease demonstrated that the amino acid sequence of a polypeptide chain contains the information needed to specify the three-dimensional structure of a protein (Anfinsen, 1973). In addition, various protein folding processes *in vivo* and *in vitro* require the assistance of chaperones. A molecular chaperone is 'a protein that binds to and stabilizes an otherwise unstable conformer of another protein and, by controlled binding and release of the substrate protein, facilitates its correct fate, be it folding, oligomeric assembly, transport to a particular subcellular compartment, or controlled switching between active/inactive conformations' (Ellis and van der Vies, 1991).

While various chaperone proteins are expressed constitutively in cells growing under normal conditions, the level of expression of many of the chaperones increase upon exposure to heat shock. The higher level of chaperone proteins minimizes or prevents protein aggregation and promotes proper folding of nascent and aggregated polypeptides (Tissieres et al., 1974). Subsequent studies revealed that the expression of these 'heat shock proteins' were also induced by other types of stress such as nutrient deprivation, oxygen radicals or viral infection. Thus molecular chaperones are also referred to as stress proteins or heat shock proteins.

Cells from bacteria to man contain distinct families of chaperones and these heat shock proteins are among the most conserved proteins known. Heat shock proteins are commonly grouped based on their molecular weight and three of the major families are hsp60, hsp70 and hsp90 (Table I).

Table I. Heat Shock Protein Families

	Component	Organism	Subcellular Localization
Hsp90	HtpG	Prokaryotes	Cytosol
	Hsp90 (Hsp83, 89)	*S. cerevisiae* Mammals	Cytosol
	Grp94/Gp96	Mammals	Endoplasmic Reticulum
Hsp70	DnaK	Prokaryotes	Cytosol
	SSA1-4 SSB1,2	*S. cerevisiae*	Cytosol
	Hsc73	Mammals	Cytosol
	KAR2	*S. cerevisiae*	Endoplasmic Reticulum
	BiP/Grp78	Mammals	Endoplasmic Reticulum
	SSC1	*S. cerevisiae*	Mitochondria
	ctHsp70	Plants	Chloroplasts
Hsp60	GroEL	Prokaryotes	Cytosol
	Hsp60	*S. cerevisiae* Mammals	Mitochondria
	Cpn60	Plants	Chloroplasts

This thesis primarily focuses on hsp70, a family of stress-inducible and constitutive proteins which are present in multiple cell compartments such as the mitochondria, endoplasmic reticulum (ER), chloroplast and cytoplasm.

Chaperoning functions of hsp70

The mechanism of hsp70-mediated protein folding has been studied extensively *in vitro*. For example, actin and luciferase proteins denatured *in vitro* with guanidium-HCl could not refold spontaneously upon dilution from denaturant. However, protein refolding could be initiated in the presence of cytosolic chaperones (Frydman and Hartl, 1996). Studies utilizing freshly translated mRNA have demonstrated that hsp70 interacts with nascent polypeptides on ribosomes to initiate their folding (Beckmann et al., 1990; Frydman et al., 1994). The translation of mRNA in the presence of chaperone proteins is thought to mimic chaperone-mediated folding of nascent proteins *in vivo*, while the interactions of chemically denatured proteins with chaperones may resemble those occurring in the cell upon exposure to various forms of stress such as heat shock. In either case, hsp70 proteins transiently associate with unfolded polypeptides--such an association can prevent aggregation of substrates, by shielding exposed hydrophobic surfaces, and assist folding, probably by decreasing the concentration of free-aggregation-prone folding intermediates.

In addition, hsp70 has multiple roles in protein translocation (Cyr and Neupert, 1996). The *S. cerevisiae* cytosolic hsp70 proteins Ssa1 and Ssa2 increase the rate of protein translocation into microsomes and mitochondria (Chirico et al., 1988; Murakami et al., 1988) and studies with conditional Ssa mutants support the involvement of cytosolic hsp70 in protein translocation into the mitochondria and the ER (Becker et al., 1996). Mitochondria import

most of their proteins from the cytosol and Ssc1, an hsp70 homologue in the mitochondrial matrix, is essential for the translocation and folding of precursor proteins (Kang et al., 1990). Mitochondrial hsp70 directly binds the polypeptide chain in transit and drives its translocation into the matrix. Furthermore, mitochondrial hsp70 is a component of the refolding machinery in the matrix (Rassow et al., 1995). The ER resident hsp70 protein BiP works in conjuction with ER membrane protein Sec63 (Hsp40 DnaJ homologue) in order to function as a ATP-dependent translocation motor and reel in the precursor protein (Lyman and Schekman, 1995; Sanders et al., 1992). BiP also mediates the ATP-dependent release of the precursor protein from the signal-sequence-receptor complex (Lyman and Schekman, 1997). Hsp70 plays a multitude of central and essential roles in the translocation of proteins across membranes.

In addition to protein folding and translocation, hsp70 is also involved in protein degradation. DnaK (hsp70 homologue) regulates its own level of expression by affecting the synthesis and stability of σ^{32}, a σ-factor which enables RNA polymerase to recognize heat shock gene promoters. When DnaK binds to σ^{32}, the susceptibility of σ^{32} to cell proteases is enhanced and σ^{32} is rapidly degraded (Sherman and Goldberg, 1992; Straus et al., 1990). However, when the level of unfolded or damaged proteins accumulate upon exposure to heat shock, the number of substrates for DnaK increase. Consequently, less DnaK is available for binding to σ^{32} and the level of σ^{32} can accumulate since it is no longer being rapidly degraded.

In various instances, molecular chaperones and heat inducible proteases act in a coordinated manner to rid the cell of abnormal proteins. In *E.coli*, the short-lived mutant form of alkaline phosphatase, PhoA61, which cannot be transported across the cell membrane, is hydrolyzed by an ATP-

dependent process that requires DnaK and the proteases La and Clp (Michaelis et al., 1986). In the mitochondrial matrix, hsp70 functions in conjunction with Pim protease in order to degrade misfolded proteins (Wagner et al., 1994). Also, prolonged interaction of immunoglobulin light chains in the ER with BiP (hsp70) leads to the degradation of the light chain polypeptide (Knittler et al., 1995). It appears that hsp70 promotes protein degradation by rendering unfolded portions of these substrates accessible to proteases.

Hsp70 structure & substrate binding

The affinity of hsp70 for substrates is tightly regulated by ATP and by co-chaperones which control the key points of the ATPase cycle (Bukau and Horwich, 1998). In hsp70-assisted folding reactions, substrates undergo repeated cycles of binding and release. The ATP-bound form of hsp70 has low affinity and fast exchange rates for substrates while the ADP-bound form of hsp70 has high affinity and slow exchange rates for substrates. The prokaryotic hsp70 system is composed of the DnaK chaperone (Hsp70 homologue) and the DnaJ (Hsp40 homologue) and GrpE co-chaperones (Szabo et al., 1994). The ATPase activity of DnaK is simulated by DnaJ and the release of ADP by hsp70 is accelerated by GrpE.

High resolution structural information on the hsp70 protein exists from crystallographic studies of (1) the ATPase domain of bovine hsc70 (Flaherty et al., 1990), (2) the ATPase domain of DnaK in complex with GrpE (Harrison et al., 1997) and (3) the C-terminal substrate binding domain of DnaK complexed with a heptapeptide substrate (Zhu et al., 1996).

Hsp70 consists of an N-terminal ATPase domain of 45kd which is composed of two structural lobes which form a cleft for ATP binding (Flaherty, et al., 1990). The ATPase domain of hsp70 binds to its cofactor GrpE

with a 1:2 stoichiometry. The dimeric GrpE complex appears to recognize the ADP-bound form of hsp70, which then leads to ADP dissociation (Harrison, et al., 1997). Unexpectedly, the GrpE dimer was found to have two long α-helices which extend beyond the ATPase domain of hsp70 and GrpE may directly affect substrate release by hsp70.

The 25kd C-terminal substrate-binding domain of hsp70 was crystallized as a complex with a heptapeptide in order to overcome the oligomerization behavior of this fragment (Zhu, et al., 1996). (Hsp70 self-associates into dimers and trimers via its C-terminal peptide binding domain (Benaroudj et al., 1997).) The hsp70 C-terminal domain is composed of a β-sandwich subdomain which binds substrates and an α-helical subdomain which appears to form a lid that encapsulates the peptide. The heptapeptide NRLLLTG was bound to hsp70 in an extended conformation an the fourth Leu residue was buried in a deep hydrophobic pocket, functioning as an anchor residue. The geometric constraints for the other residues were less restrictive although hydrophobic residues in the central portion of the substrate is most compatible with the binding site.

Various other studies have demonstrated the ability of hsp70 to bind short synthetic peptides (Flynn et al., 1991; Gragerov and Gottesman, 1994; Roman et al., 1994) and the peptide binding specificity has been examined utilizing randomly synthesized heptapeptides (Flynn, et al., 1991), bacteriophage peptide-display libraries (Blond-Elguindi et al., 1993) and a library of 13-mer peptides representing 37 complete sequences of proteins (Rudiger et al., 1997). Peptides which lack aromatic or large hydrophobic residues bind poorly to hsp70 (Blond-Elguindi, et al., 1993; Flynn, et al., 1991) and the binding motif of DnaK is composed of a hydrophobic core of 4-5

residues (Rudiger, et al., 1997). These peptide binding studies are compatible with the crystal structure of the substrate-binding domain of hsp70.

In summary, hsp70 binds to linear hydrophobic protein segments and by binding to other cofactors, hsp70 is involved in chaperoning functions such as and (1) folding proteins in the mitochondria, endoplasmic reticulum and cytosol; (2) degrading unstable proteins and the bacterial heat shock transcription factor σ^{32}; (3) guiding translocating proteins into cellular compartments such as the mitochondria, and the ER.

Section I

Chapter 2. Heat shock proteins are among the major targets of the immune response to many bacterial and parasitic pathogens.

Humans are exposed to a large number and variety of pathogenic and non-pathogenic microorganisms. Our bodies provide niches for a multitude of microbes that do not typically cause disease. For example, approximately 10^6 cocci and diptheroides reside on one cubic centimeter of human skin (Leyden et al., 1991) and 1 ml of saliva contains about 10^8 microbes (Rosebury, 1962). We are also continuously confronted by pathogenic organisms of substantial diversity.

To protect us from these microorganisms, the immune system has evolved to recognize a remarkable variety of antigenic determinants. Despite this capacity, the immune system appears to devote considerable attention to the members of one family of proteins, the ubiquitous heat shock proteins (HSPs) (Kaufmann, 1990; Kaufmann and Schoel, 1994b; Murray and Young, 1992; Young et al., 1990b; Young, 1990a). The HSPs are among the major targets of the immune response to most bacterial and parasitic pathogens. Humoral and cellular immune responses to HSPs have been observed following exposure to a broad spectrum of infectious agents, including gram positive and gram negative bacteria, fungi, helminths and protozoa (Table II).

Table II
Pathogens induce immune responses to HSPs

Infectious agent	Disease	HSP	References
Bacteria			
Bordetella pertussis	pertussis	hsp70, hsp60	Del Giudice et al., 1993
Borrelia burgdorferi	Lyme disease	hsp60	Hansen et al., 1988
Brucella abortus	brucellosis	hsp60	Roop et al., 1992
Chlamydia trachomatis	blinding trachoma	hsp70, hsp60	Taylor et al., 1990; Cerrone et al., 1991; Zhong and Brunham, 1992
Coxiella burnetti	Q fever	hsp60	Vodkin and Williams, 1988
Helicobacter pylori	gastritis	hsp60, hsp10	Suerbaum, 1994; Ferrero et al., 1995
Legionella pneumophila	Legionnaires' disease	hsp60	Hoffman et al., 1990
Mycobacterium leprae	leprosy	hsp70, hsp60, small hsps	Mehra et al., 1992; Young et al., 1988
Mycobacterium tuberculosis	tuberculosis	hsp70, hsp60, small hsps	Young et al., 1988; Shinnick et al., 1988; Baird et al., 1988
Treponema pallidum	syphilis	hsp60	Hindersson et al., 1987
Fungi			
Aspergillus fumigatus	aspergillosis	hsp60	Kumar et al., 1993
Candida albicans	candidasis	hsp90	Matthews et al, 1987; Matthews and Burnie 1989
Histoplasma capsulatum	histoplasmosis	hsp60, hsp70	Gomez et al., 1991a; 1992; 1995
Helminths			
Brugia malayi	lymphatic filariasis	hsp70	Selkirk et al., 1989
Onchocerca volvus	ocular filariasis	hsp70	Rothstein et al., 1989
Schistosoma mansoni	schistosomiasis	hsp90, hsp70, small hsps	Johnson et al., 1989; Hedstrom et al., 1987; Nene et al., 1986
Schistosoma japonicum	schistosomiasis	hsp70	Scallon et al., 1987; Hedstrom et al., 1988
Protozoa			
Leishmania braziliensis	leishmaniasis	hsp70	Levy Yeyati et al., 1992
Leishmania donovani	visceral leishmaniasis	hsp90, hsp70	MacFarlane et al., 1990; de Andrade et al., 1992
Plasmodium falciparum	malaria	hsp70	Mattei et al., 1989; Renia et al., 1990
Trypanosoma cruzi	Chagas' disease	hsp70	Levy Yeyati et al., 1992; Requena et al. 1993

The immune responses to HSPs elicited by mycobacterial pathogens have been particularly well-studied. Exposure to *Mycobacterium tuberculosis* or *Mycobacterium leprae* leads to humoral and cellular immune responses to hsp70, hsp60 and small hsps (18kD, 14kD, 10kD) (Adams et al., 1994; Husson and Young, 1987; Mehra, et al., 1992; Nerland et al., 1988; Verbon et al., 1992; Young, et al., 1988). The cellular responses to mycobacterial HSPs are profound; limiting dilution analysis indicates that 20% of the murine $CD4^+$ T lymphocytes that recognize mycobacterial antigens are directed against hsp60 alone (Kaufmann et al., 1987). The high frequency with which human $CD4^+$ T cell clones directed against mycobacterial hsp70 and hsp60 have been detected suggests that these HSPs are also major targets of the cellular response in humans (Munk et al., 1988). Limiting dilution analysis of human T lymphocytes from a tuberculoid leprosy patient as well as a patient contact revealed that one third of *M. leprae* reactive T cells were directed against hsp10 (Mehra, et al., 1992).

The powerful antigenic nature of HSPs is emphasized by evidence that mammals are capable of recognizing multiple B and T cell epitopes in these proteins. Murine and human B cell epitopes have been mapped in HSPs from *M. tuberculosis, M. leprae, Trypanosoma cruzi* and *Plasmodium falciparum* (Mattei, et al., 1989; Matthews et al., 1991; Mehra et al., 1986; Requena, et al., 1993; Richman et al., 1989; Thole et al., 1988). These mapping data indicate that B cells can recognize many portions of the hsp70 and hsp60 protein molecules. Murine and human T cell epitopes have been mapped most extensively for mycobacterial hsp60 and hsp70 (Adams, et al., 1994; Lamb et al., 1987; Munk et al., 1990; Oftung et al., 1994; Van Schooten et al., 1988). This evidence indicates that mycobacterial HSPs can be presented in the

context of multiple MHC haplotypes, and that T cell epitopes can be found throughout these HSPs.

HSPs derived from a variety of bacterial and fungal pathogens have been found to stimulate protective immunity in animal models when used as subunit vaccines. For example, vaccination of mice with syngenic J774 macrophage cells expressing mycobacterial hsp60 afforded remarkable protection against *M. tuberculosis* (Silva and Lowrie, 1994a). In mice vaccinated with J774-hsp60, 100 times fewer *M. tuberculosis* CFUs could be recovered from the liver 5 weeks after challenge, compared to unvaccinated mice. Hsp60 specific T cells cloned from the vaccinated mice could adoptively transfer protection to non-vaccinated mice (Silva et al., 1994b). Vaccination of mice with mycobacterial hsp60 was also effective when administered to mice as a naked DNA vaccine (Bonato et al., 1998; Lowrie et al., 1994)

As shown in Table III, the immune responses against HSPs confer protection against a broad range of pathogens. Although there is some concern with using HSPs in vaccine formulations due to their highly conserved nature and homology with self-HSPs, it must be emphasized that healthy individuals are routinely stimulated to respond to HSPs without causing autoimmunity. For example, the trivalent vaccine against tetanus, diphtheria and pertussis, which is routinely administered to infants, induces anti-hsp70 immune responses (Del Giudice, et al., 1993). Live BCG, which contains substantial amounts of hsp70 and hsp60, has been used to immunize 80% of the world's children against tuberculosis. Thus, current knowledge suggests that the inclusion of HSPs in vaccines against a broad spectrum of infectious diseases would be both safe and beneficial.

Table III
HSPs can elicit protective immune responses

Infectious agent	HSP	Animal model	References
Bacteria			
Helicobacter pylori	hsp60, hsp10	mouse	Ferrero et al., 1995
Legionella pneumophila	hsp60	guinea pig	Blander and Horwitz, 1993
Mycobacterium leprae	hsp60, hsp10	mouse	Gelber et al., 1992;1994
Mycobacterium tuberculosis	hsp70	mouse, guinea pig	Pal and Horwitz, 1992; Hubbard et al., 1992; Andersen 1994; Horwtiz et al., 1995
Mycobacterium tuberculosis	hsp60	mouse	Silva et al., 1994a, 1994b; Lowrie et al., 1994
Yersinia enterocolitica	hsp60	mouse	Noll et al., 1994
Fungi			
Candida albicans	hsp90	mouse	Matthews et al, 1991
Histoplasma capsulatum	hsp60, hsp70	mouse	Gomez et al., 1991a; 1991b; 1992; 1995

Section I References

Adams, E., W. Britton, A. Morgan, S. Sergeantson and A. Basten. 1994. Individuals from different populations identify multiple and diverse T-cell determinants on mycobacterial HSP70. *Scand J Immunol* 39:588-96.

Andersen, P. 1994. Effective vaccination of mice against Mycobacterium tuberculosis infection with a soluble mixture of secreted mycobacterial proteins. *Infect Immun* 62:2536-44.

Anfinsen, C. B. 1973. Principles that govern the folding of protein chains. *Science* 181:223-30.

Baird, P. N., L. M. Hall and A. R. Coates. 1988. A major antigen from Mycobacterium tuberculosis which is homologous to the heat shock proteins groES from E. coli and the htpA gene product of Coxiella burneti. *Nucleic Acids Res* 16:9047.

Becker, J., W. Walter, W. Yan and E. A. Craig. 1996. Functional interaction of cytosolic hsp70 and a DnaJ-related protein, Ydj1p, in protein translocation in vivo. *Mol Cell Biol* 16:4378-86.

Beckmann, R. P., L. E. Mizzen and W. J. Welch. 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 248:850-4.

Benaroudj, N., B. Fouchaq and M. M. Ladjimi. 1997. The COOH-terminal peptide binding domain is essential for self-association of the molecular chaperone HSC70. *J Biol Chem* 272:8744-51.

Blander, S. J. and M. A. Horwitz. 1993. Major cytoplasmic membrane protein of Legionella pneumophila, a genus common antigen and member of the hsp 60 family of heat shock proteins, induces protective immunity in a guinea pig model of Legionnaires' disease. *J Clin Invest* 91:717-23.

Blond-Elguindi, S., S. E. Cwirla, W. J. Dower, R. J. Lipshutz, S. R. Sprang, J. F. Sambrook and M. J. Gething. 1993. Affinity panning of a library of peptides displayed on bacteriophages reveals the binding specificity of BiP. *Cell* 75:717-28.

Bonato, V. L. D., V. M. F. Lima, R. E. Tascon, D. B. Lowrie and C. L. Silva. 1998. Identification and characterization of protective T cells in hsp65 DNA-vaccinated and *Mycobacterium tuberculosis*-infected mice. *Infection and Immunity* 66:169-175.

Bukau, B. and A. L. Horwich. 1998. The Hsp70 and Hsp60 chaperone machines. *Cell* 92:351-366.

Cerrone, M. C., J. J. Ma and R. S. Stephens. 1991. Cloning and sequence of the gene for heat shock protein 60 from Chlamydia trachomatis and immunological reactivity of the protein. *Infect Immun* 59:79-90.

Chirico, W. J., M. G. Waters and G. Blobel. 1988. 70K heat shock related proteins stimulate protein translocation into microsomes. *Nature* 332:805-10.

Cyr, D. M. and W. Neupert. 1996. Roles for hsp70 in protein translocation across membranes of organelles. In *Stress-Inducible Cellular Responses*, U. Feige, R. I. Morimoto, I. Yahara and B. S. Polla, eds. Birkhauser Verlag, Basel, p. 25-40.

de Andrade, C. R., L. V. Kirchhoff, J. E. Donelson and K. Otsu. 1992. Recombinant Leishmania Hsp90 and Hsp70 are recognized by sera from visceral leishmaniasis patients but not Chagas' disease patients. *J Clin Microbiol* 30:330-5.

Del Giudice, G., A. Gervaix, P. Costantino, C. A. Wyler, C. Tougne, E. R. de Graeff-Meeder, J. Van Embden, R. Van der Zee, L. Nencioni, R. Rappuoli, S. Suter and P. H. Lambert. 1993. Priming to heat shock proteins in infants vaccinated against pertussis. *J Immunol* 150:2025-32.

Ellis, R. J. and S. M. van der Vies. 1991. Molecular chaperones. *Annu Rev Biochem* 60:321-47.

Ferrero, R. L., J.-M. Thiberge, I. Kansau, N. Wuscher, M. Huerre and A. Labigne. 1995. The GroES homolog of Helicobacter pylori confers protective immunity against mucosal infection in mice. *Proc Natl Acad Sci U S A* 92:6499-6503.

Flaherty, K. M., C. DeLuca-Flaherty and D. B. McKay. 1990. Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. *Nature* 346:623-628.

Flynn, G. C., J. Pohl, M. T. Flocco and J. E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature* 353:726-30.

Frydman, J. and F. U. Hartl. 1996. Principles of chaperone-assisted protein folding: differences between in vitro and in vivo mechanisms. *Science* 272:1497-502.

Frydman, J., E. Nimmesgern, K. Ohtsuka and F. U. Hartl. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370:111-7.

Gelber, R. H., V. Mehra, B. Bloom, L. P. Murray, P. Siu, M. Tsang and P. J. Brennan. 1994. Vaccination with pure Mycobacterium leprae proteins inhibits M. leprae multiplication in mouse footpads. *Infect Immun* 62:4250-5.

Gelber, R. H., L. Murray, P. Siu and M. Tsang. 1992. Vaccination of mice with a soluble protein fraction of Mycobacterium leprae provides consistent and long-term protection against M. leprae infection. *Infect Immun* 60:1840-4.

Gomez, A. M., J. C. Rhodes and G. Deepe Jr. 1991a. Antigenicity and immunogenicity of an extract from the cell wall and cell membrane of Histoplasma capsulatum yeast cells. *Infect Immun* 59:330-6.

Gomez, F. J., R. Allendoerfer and G. S. Deepe. 1995. Vaccination with Recombinant Heat Shock Protein 60 from Histoplasma capsulatum Protects Mice against Pulmonary Histoplasmosis. *Infection and Immunity* 63:2587-2595.

Gomez, F. J., A. M. Gomez and G. Deepe Jr. 1991b. Protective efficacy of a 62-kilodalton antigen, HIS-62, from the cell wall and cell membrane of Histoplasma capsulatum yeast cells. *Infect Immun* 59:4459-64.

Gomez, F. J., A. M. Gomez and G. Deepe Jr. 1992. An 80-kilodalton antigen from Histoplasma capsulatum that has homology to heat shock protein 70 induces cell-mediated immune responses and protection in mice. *Infect Immun* 60:2565-71.

Gragerov, A. and M. E. Gottesman. 1994. Different peptide binding specificities of hsp70 family members. *J Mol Biol* 241:133-5.

Hansen, K., J. M. Bangsborg, H. Fjordvang, N. S. Pedersen and P. Hindersson. 1988. Immunochemical characterization of and isolation of the gene for a Borrelia burgdorferi immunodominant 60-kilodalton antigen common to a wide range of bacteria. *Infect Immun* 56:2047-53.

Harrison, C. J., M. Hayer-Hartl, M. Di Liberto, F. Hartl and J. Kuriyan. 1997. Crystal structure of the nucleotide exchange factor GrpE bound to the ATPase domain of the molecular chaperone DnaK. *Science* 276:431-5.

Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian and G. Newport. 1987. A major immunogen in Schistosoma mansoni infections is homologous to the heat-shock protein Hsp70. *J Exp Med* 165:1430-5.

Hedstrom, R., J. Culpepper, V. Schinski, N. Agabian and G. Newport. 1988. Schistosome heat-shock proteins are immunologically distinct host-like antigens. *Mol Biochem Parasitol* 29:275-82.

Hindersson, P., J. D. Knudsen and N. H. Axelsen. 1987. Cloning and expression of treponema pallidum common antigen (Tp-4) in Escherichia coli K12. *J Gen Microbiol* 133:587-96.

Hoffman, P. S., L. Houston and C. A. Butler. 1990. Legionella pneumophila htpAB heat shock operon: nucleotide sequence and expression of the 60-kilodalton antigen in L. pneumophila-infected HeLa cells. *Infect Immun* 58:3380-7.

Horwitz, M. A., B. W. Lee, B. J. Dillon and G. Harth. 1995. Protective immunity against tuberculosis induced by vaccination with major extracellular proteins of Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* 92:1530-4.

Hubbard, R. D., C. M. Flory and F. M. Collins. 1992. Immunization of mice with mycobacterial culture filtrate proteins. *Clin Exp Immunol* 87:94-8.

Husson, R. N. and R. A. Young. 1987. Genes for the major protein antigens of Mycobacterium tuberculosis: the etiologic agents of tuberculosis and leprosy share an immunodominant antigen. *Proc Natl Acad Sci U S A* 84:1679-83.

Johnson, K. S., K. Wells, J. V. Bock, V. Nene, D. W. Taylor and J. S. Cordingley. 1989. The 86-kilodalton antigen from Schistosoma mansoni is a heat-shock protein homologous to yeast HSP-90. *Mol Biochem Parasitol* 36:19-28.

Kang, P. J., J. Ostermann, J. Shilling, W. Neupert, E. A. Craig and N. Pfanner. 1990. Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* 348:137-43.

Kaufmann, S. H. 1990. Heat shock proteins and the immune response. *Immunol Today* 11:129-36.

Kaufmann, S. H., U. Vath, J. E. Thole, J. D. Van Embden and F. Emmrich. 1987. Enumeration of T cells reactive with Mycobacterium tuberculosis organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur J Immunol* 17:351-7.

Kaufmann, S. H. E. and B. Schoel. 1994b. Heat shock proteins as antigens in immunity against infection and self. In *The biology of heat shock proteins and molecular chaperones*, R. I. Morimoto, A. Tissieres and C. Georgopoulos, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 495-531.

Knittler, M. R., S. Dirks and I. G. Haas. 1995. Molecular chaperones involved in protein degradation in the endoplasmic reticulum: quantitative interaction of the heat shock cognate protein BiP with partially folded immunoglobulin light chains that are degraded in the endoplasmic reticulum. *Proc Natl Acad Sci U S A* 92:1764-8.

Kumar, A., L. V. Reddy, A. Sochanik and V. P. Kurup. 1993. Isolation and characterization of a recombinant heat shock protein of Aspergillus fumigatus. *J Allergy Clin Immunol* 91:1024-30.

Lamb, J. R., J. Ivanyi, A. D. Rees, J. B. Rothbard, K. Howland, R. A. Young and D. B. Young. 1987. Mapping of T cell epitopes using recombinant antigens and synthetic peptides. *Embo J* 6:1245-9.

Levy Yeyati, P., S. Bonnefoy, G. Mirkin, A. Debrabant, S. Lafon, A. Panebra, E. Gonzalez-Cappa, J. P. Dedet, M. Hontebeyrie-Joskowicz and M. J. Levin. 1992. The 70-kDa heat-shock protein is a major antigenic determinant in human Trypanosoma cruzi/Leishmania braziliensis braziliensis mixed infection. *Immunol Lett* 31:27-33.

Leyden, J. J., K. M. Nordstrom and K. J. McGinley. 1991. Cutaneous Microbiology. In *Physiology, biochemistry, and molecular biology of the skin*, L. A. Goldsmith, eds. Oxford university press, New York, p. 1403-14213.

Lowrie, D. B., R. E. Tascon, M. J. Colston and C. L. Silva. 1994. Towards a DNA vaccine against tuberculosis. *Vaccine* 12:1537-1540.

Lyman, S. K. and R. Schekman. 1995. Interaction between BiP and Sec63p is required for the completion of protein translocation into the ER of Saccharomyces cerevisiae. *J Cell Biol* 131:1163-71.

Lyman, S. K. and R. Schekman. 1997. Binding of secretory precursor polypeptides to a translocon subcomplex is regulated by BiP. *Cell* 88:85-96.

MacFarlane, J., M. L. Blaxter, R. P. Bishop, M. A. Miles and J. M. Kelly. 1990. Identification and characterisation of a Leishmania donovani antigen belonging to the 70-kDa heat-shock protein family. *Eur J Biochem* 190:377-84.

Mattei, D., A. Scherf, O. Bensaude and L. P. da Silva. 1989. A heat shock-like protein from the human malaria parasite Plasmodium falciparum induces autoantibodies. *Eur J Immunol* 19:1823-8.

Matthews, R. C. and J. P. Burnie. 1989. Cloning of a DNA sequence encoding a major fragment of the 47 kilodalton stress protein homologue of Candida albicans. *FEMS Microbiol Letts* 60:25.

Matthews, R. C., J. P. Burnie, D. Howat, T. Rowland and F. Walton. 1991. Autoantibody to heat-shock protein 90 can mediate protection against systemic candidosis. *Immunology* 74:20-4.

Matthews, R. C., J. P. Burnie and S. Tabaqchali. 1987. Isolation of immunodominant antigens from sera of patients with systemic candidiasis and characterization of serological response to Candida albicans. *J Clin Microbiol* 25:230-7.

Mehra, V., B. R. Bloom, A. C. Bajardi, C. L. Grisso, P. A. Sieling, D. Alland, J. Convit, X. D. Fan, S. W. Hunter, P. J. Brennan, T. H. Rea and R. L. Modlin. 1992. A major T cell antigen of Mycobacterium leprae is a 10-kD heat-shock cognate protein. *J Exp Med* 175:275-84.

Mehra, V., D. Sweetser and R. A. Young. 1986. Efficient mapping of protein antigenic determinants. *Proc Natl Acad Sci U S A* 83:7013-7.

Michaelis, S., J. F. Hunt and J. Beckwith. 1986. Effects of signal sequence mutations on the kinetics of alkaline phosphatase export to the periplasm in Escherichia coli. *J Bacteriol* 167:160-7.

Munk, M. E., B. Schoel and S. H. Kaufmann. 1988. T cell responses of normal individuals towards recombinant protein antigens of Mycobacterium tuberculosis. *Eur J Immunol* 18:1835-8.

Munk, M. E., T. M. Shinnick and S. H. Kaufmann. 1990. Epitopes of the mycobacterial heat shock protein 65 for human T cells comprise different structures. *Immunobiology* 180:272-7.

Murakami, H., D. Pain and G. Blobel. 1988. 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria. *J Cell Biol* 107:2051-7.

Murray, P. J. and R. A. Young. 1992. Stress and immunological recognition in host-pathogen interactions. *J Bacteriol* 174:4193-6.

Nene, V., D. W. Dunne, K. S. Johnson, D. W. Taylor and J. S. Cordingley. 1986. Sequence and expression of a major egg antigen from Schistosoma mansoni. Homologies to heat shock proteins and alpha-crystallins. *Mol Biochem Parasitol* 21:179-88.

Nerland, A. H., A. S. Mustafa, D. Sweetser, T. Godal and R. A. Young. 1988. A protein antigen of Mycobacterium leprae is related to a family of small heat shock proteins. *J Bacteriol* 170:5919-21.

Noll, A., A. Roggenkamp, J. Heesemann and I. B. Autenrieth. 1994. Protective role for heat shock protein-reactive alpha beta T cells in murine yersiniosis. *Infect Immun* 62:2784-91.

Oftung, F., A. Geluk, K. E. Lundin, R. H. Meloen, J. E. Thole, A. S. Mustafa and T. H. Ottenhoff. 1994. Mapping of multiple HLA class II-restricted T-cell epitopes of the mycobacterial 70-kilodalton heat shock protein. *Infect Immun* 62:5411-8.

Pal, P. G. and M. A. Horwitz. 1992. Immunization with extracellular proteins of Mycobacterium tuberculosis induces cell-mediated immune responses and substantial protective immunity in a guinea pig model of pulmonary tuberculosis. *Infect Immun* 60:4781-92.

Rassow, J., K. Mohrs, S. Koidl, I. B. Barthelmess, N. Pfanner and M. Tropschug. 1995. Cyclophilin 20 is involved in mitochondrial protein folding in cooperation with molecular chaperones Hsp70 and Hsp60. *Mol Cell Biol* 15:2654-62.

Renia, L., D. Mattei, J. Goma, S. Pied, P. Dubois, F. Miltgen, A. Nussler, H. Matile, F. Menegaux, M. Gentilini and a. et. 1990. A malaria heat-shock-like determinant expressed on the infected hepatocyte surface is the target of antibody-dependent cell-mediated cytotoxic mechanisms by nonparenchymal liver cells. *Eur J Immunol* 20:1445-9.

Requena, J. M., M. Soto, F. Guzman, A. Maekelt, O. Noya, M. E. Patarroyo and C. Alonso. 1993. Mapping of antigenic determinants of the T. cruzi hsp70 in chagasic and healthy individuals. *Mol Immunol* 30:1115-21.

Richman, S. J., T. S. Vedvick and R. T. Reese. 1989. Peptide mapping of conformational epitopes in a human malarial parasite heat shock protein. *J Immunol* 143:285-92.

Roman, E., C. Moreno and D. Young. 1994. Mapping of Hsp70-binding sites on protein antigens. *Eur J Biochem* 222:65-73.

Roop, R. 2., M. L. Price, B. E. Dunn, S. M. Boyle, N. Sriranganathan and G. G. Schurig. 1992. Molecular cloning and nucleotide sequence analysis of the gene encoding the immunoreactive Brucella abortus Hsp60 protein, BA60K. *Microb Pathog* 12:47-62.

Rosebury, T. 1962. Microorganisms indigenous to man. McGraw-hill book co., inc., New York. 310-350 pp.

Rothstein, N. M., G. Higashi, J. Yates and T. V. Rajan. 1989. Onchocerca volvulus heat shock protein 70 is a major immunogen in amicrofilaremic individuals from a filariasis-endemic area. *Mol Biochem Parasitol* 33:229-35.

Rudiger, S., L. Germeroth, J. Schneider-Mergener and B. Bukau. 1997. Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *Embo J* 16:1501-7.

Sanders, S. L., K. M. Whitfield, J. P. Vogel, M. D. Rose and R. W. Schekman. 1992. Sec61p and BiP directly facilitate polypeptide translocation into the ER. *Cell* 69:353-65.

Scallon, B. J., B. J. Bogitsh and C. E. Carter. 1987. Cloning of a Schistosoma japonicum gene encoding a major immunogen recognized by hyperinfected rabbits. *Mol Biochem Parasitol* 24:237-45.

Selkirk, M. E., D. A. Denham, F. Partono and R. M. Maizels. 1989. Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. *J Immunol* 143:299-308.

Sherman, M. and A. L. Goldberg. 1992. Involvement of the chaperonin dnaK in the rapid degradation of a mutant protein in Escherichia coli. *Embo J* 11:71-7.

Shinnick, T. M., M. H. Vodkin and J. C. Williams. 1988. The Mycobacterium tuberculosis 65-kilodalton antigen is a heat shock protein which corresponds to common antigen and to the Escherichia coli GroEL protein. *Infect Immun* 56:446-51.

Silva, C. L. and D. B. Lowrie. 1994a. A single mycobacterial protein (hsp 65) expressed by a transgenic antigen-presenting cell vaccinates mice against tuberculosis. *Immunology* 82:244-8.

Silva, C. L., M. F. Silva, R. C. Pietro and D. B. Lowrie. 1994b. Protection against tuberculosis by passive transfer with T-cell clones recognizing mycobacterial heat-shock protein 65. *Immunology* 83:341-6.

Straus, D., W. Walter and C. A. Gross. 1990. DnaK, DnaJ, and GrpE heat shock proteins negatively regulate heat shock gene expression by controlling the synthesis and stability of sigma 32. *Genes Dev* 4:2202-9.

Suerbaum, S., J. M. Thiberge, I. Kansau, R. L. Ferrero and A. Labigne. 1994. Helicobacter pylori hspA-hspB heat-shock gene cluster: nucleotide sequence, expression, putative function and immunogenicity. *Mol Microbiol* 14:959-74.

Szabo, A., T. Langer, H. Schroder, J. Flanagan, B. Bukau and F. U. Hartl. 1994. The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE. *Proc Natl Acad Sci U S A* 91:10345-9.

Taylor, H. R., I. W. Maclean, R. C. Brunham, S. Pal and J. Wittum-Hudson. 1990. Chlamydial heat shock proteins and trachoma. *Infect Immun* 58:3061-3.

Thole, J. E., W. C. van Schooten, W. J. Keulen, P. W. Hermans, A. A. Janson, R. R. de Vries, A. H. Kolk and J. D. van Embden. 1988. Use of recombinant antigens expressed in Escherichia coli K-12 to map B-cell and T-cell epitopes on the immunodominant 65-kilodalton protein of Mycobacterium bovis BCG. *Infect Immun* 56:1633-40.

Tissieres, A., H. K. Mitchell and U. M. Tracy. 1974. Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. *J Mol Biol* 84:389-98.

Van Schooten, W. C., T. H. Ottenhoff, P. R. Klatser, J. Thole, R. R. De Vries and A. H. Kolk. 1988. T cell epitopes on the 36K and 65K Mycobacterium leprae antigens defined by human T cell clones. *Eur J Immunol* 18:849-54.

Verbon, A., R. A. Hartskeerl, A. Schuitema, A. H. Kolk, D. B. Young and R. Lathigra. 1992. The 14,000-molecular-weight antigen of Mycobacterium tuberculosis is related to the alpha-crystallin family of low-molecular-weight heat shock proteins. *J Bacteriol* 174:1352-9.

Vodkin, M. H. and J. C. Williams. 1988. A heat shock operon in Coxiella burnetti produces a major antigen homologous to a protein in both mycobacteria and Escherichia coli. *J Bacteriol* 170:1227-34.

Wagner, I., H. Arlt, L. van Dyck, T. Langer and W. Neupert. 1994. Molecular chaperones cooperate with PIM1 protease in the degradation of misfolded proteins in mitochondria. *Embo J* 13:5135-45.

Young, D., R. Lathigra, R. Hendrix, D. Sweetser and R. A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A* 85:4267-70.

Young, D. B., A. Mehlert and D. F. Smith. 1990b. Stress proteins and Infectious diseases. In *Stress proteins in biology and medicine*, R. I. Morimoto, A. Tissieres and C. Georgopoulos, eds. Cold Spring Harbor Laboratory, Cold Spring Harobr, N.Y., p. 131-165.

Young, R. A. 1990a. Stress proteins and immunology. *Annu Rev Immunol* 8:401-20.

Zhong, G. and R. C. Brunham. 1992. Antibody responses to the chlamydial heat shock proteins hsp60 and hsp70 are H-2 linked. *Infect Immun* 60:3143-9.

Zhu, X., X. Zhao, W. F. Burkholder, A. Gragerov, C. M. Ogata, M. E. Gottesman and W. A. Hendrickson. 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* 272:1606-1614.

Section II

Generation of Humoral Immune Responses

Section II: Generation of Humoral Immune Responses

The powerful chaperoning and immunological features of HSPs have led to their experimental use as immunologic carrier molecules. In chapter 3, I will describe the concepts behind immunological carrier proteins and adjuvants, which are typically co-administered with an antigen of interest. I will then discuss the adjuvant-free carrier effects of hsp60 and hsp70 and describe my work with hsp70 fusion proteins in chapter 4. The work described in chapter 4 has been published as: Suzue, K. and Young, R.A., Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. J. Immunol. 156: 873-879 (1996).

Section II

Chapter 3. Immunological carrier proteins and adjuvants

Carrier proteins

Many polysaccharides and simple chemical compounds are inherently non-immunogenic and fail to elicit strong antibody responses. Landsteiner observed that these substances, "haptens", react *in vitro* with antibodies but do not have the capacity to elicit antibodies *in vivo* (Landsteiner, 1936). However, if the hapten was administered in combination with a "carrier" protein, antibodies to the hapten could be generated.

Ovary and Benacerraf demonstrated that the same carrier protein used in the primary immunization must be used in the subsequent immunization in order to elicit a secondary immune response to the hapten (Ovary and Benacerraf, 1963). The hapten and carrier had to be physically linked and within this conjugate molecule, cells recognized one antigenic determinant on the hapten and a second determinant on the carrier (Mitchison, 1971a; Rajewsky et al., 1969). It was determined that in the generation of an antibody response, two distinct types of cells were involved: bone marrow derived lymphocytes (B cells) and thymus derived lymphocytes (T cells) (Claman et al., 1966; Davies et al., 1967; Miller and Mitchell, 1967). Involvement of B and T cell cooperation in the "carrier effect" was demonstrated by adoptive transfer experiments in which one mouse was injected with the hapten, a second mouse was injected with the carrier and a third mouse was irradiated and received hapten-primed B cells and carrier-primed T cells (Mitchison, 1971b; Raff, 1970). The B and T cells could collaborate to generate an antibody

response only if they were obtained from syngeneic mice (Katz et al., 1973; Kindred and Shreffler, 1972).

The "carrier effect" is believed to occur in the following manner. When animals are primed with a hapten-carrier preparation, and then exposed to a second dose, hapten-specific B cells recognize, internalize and process the hapten-carrier conjugate. The B cell can then present peptides derived from the carrier molecule on its surface in the context of an MHC molecule. This MHC/peptide complex is bound by a carrier-primed T cell, leading to the directed release of cytokines by the T cell to the B cell. These soluble factors stimulate the B cell to proliferate, differentiate and secrete antibodies. In this thesis, a "carrier" will refer to a molecule containing T cell epitopes which, when covalently linked to a second molecule, help to elicit and enhance immune responses against the second molecule.

Carriers are an important component of some human vaccines. Tetanus toxoid (TT), diphtheria toxoid (DT) and neisseria outer membrane proteins are the carrier molecules used in the various *Haemophilus influenzae* vaccines licensed for use in humans (Smith et al., 1989). The principal virulence determinant of *H. influenzae* type b, a repeating polymer of ribose, ribitol and phosphate, is classified as a T-independent antigen because it elicits predominantly IgM antibodies and fails to elicit secondary antibody responses. In the *H. influenzae* vaccines, the capsular polysaccharide antigen had to be conjugated to a carrier in order to elicit the high levels of anti-polysaccharide antibodies necessary for protective immunity in young children (Robbins and Schneerson, 1990).

The choice of an appropriate carrier protein is of primary importance for optimizing the response to an attached antigen. In certain circumstances, previous exposure to a carrier protein alone will prevent the elicitation of an

immune response to a new antigenic epitope presented on the same carrier. Priming animals with KLH before exposure to DNP-KLH (dinitrophenyl-keyhole limpet hemocyanin) reduced the IgG antibody response against DNP (Herzenberg et al., 1980). Priming with tetanus toxoid (TT) or with bovine serum albumin (BSA) strongly reduced the anti-peptide antibody response when animals were immunized with peptides conjugated to the homologous carrier (Lise et al., 1987). This phenomenon, termed epitope-specific suppression is observed with some but not all carrier-antigen combinations. As will be explained shortly, hsp60 or hsp70 proteins do not cause epitope-specific suppression when they are utilized as carrier proteins (Barrios et al., 1992). As the number of vaccines containing the same carrier increases, the concern for epitope suppression and competition also increases and the selection of optimal carrier proteins will continue to grow in importance.

Adjuvants

The immune response to an antigen of interest can also be enhanced by use of an adjuvant (derived from the Latin word adjuvare-to aid). Indeed, adjuvants are often necessary to elicit desired immune responses (Geerligs et al., 1989; Kenney et al., 1989). In contrast to a carrier, an adjuvant does not need to be covalently coupled to the antigen to perform its function. Instead, the adjuvant and antigen are adsorbed or mixed together and are co-administered (Nicklas, 1992). In general, adjuvants function by slowly releasing the antigen, thereby acting as a long-lived antigen reservoir (called the depot effect), and by causing general inflammation at the injection site and thus recruiting immunological mediators such as macrophages (Cox and Coulter, 1997). Many adjuvants containing bacterial components, oils and various chemicals have been described (Edelman and Tacket, 1990).

Alum, which contains the aluminum salts $Al(OH)_3$ and $AlPO_4$, is currently the only adjuvant licensed for use in humans and is included in vaccines against diphtheria, tetanus, pertussis, *Haemophilus influenza* and hepatitis B. Alum has been widely used in human and veterinary vaccines since 1930 since it has the capability of stimulating strong T helper type 2 responses in certain circumstances, which result in high antibody levels (Mark et al., 1995). However, alum is poor at stimulating cell mediated responses and is not effective in the induction of humoral responses against certain antigens (Altman and Dixon, 1989). Clinical trials have failed to demonstrate that alum enhances the immunogenicity of purified influenza virus hemagglutinin and for this reason, alum is not included in the influenza vaccine formulation (Davenport et al., 1968). A considerable effort is thus being made to develop new safe and effective adjuvants for use in man (Audibert and Lise, 1993; Dintzis, 1992; Lussow et al., 1990a).

Adjuvant-free carrier effect of hsp60 and hsp70 proteins

Purified protein derivative (PPD), prepared from mycobacterial culture supernatant, is a protein mix which contains hsp60 and hsp70 and elicits a delayed type hypersensitivity reaction in individuals previously exposed to mycobacteria. The powerful immunostimulatory properties of PPD also suggested that it might have some utility as an immunological carrier. Indeed, when PPD was cross-linked to small chemical haptens or peptides, the conjugates elicited a strong antibody response against the attached molecules (Lachmann and Amos, 1970; Lachmann et al., 1986). In addition, a PPD-tumor cell conjugate has been found to enhance the immune response to tumor cells (Lachmann and Sikora, 1978). For PPD to be an effective carrier, physical linkage of PPD to the antigen was crucial and carrier priming

with bacille Calmette-Guerin (BCG) was necessary. The powerful carrier effect of PPD was also evident in comparative studies using various conjugates administered with Freund's adjuvant; these studies demonstrated that PPD was a more effective carrier than bovine serum albumin or keyhole limpet hemacyanin (Lachmann, et al., 1986). PPD has been shown to be an effective carrier in the absence of adjuvant. When the synthetic malarial peptide $(NANP)_{40}$, an epitope from the *Plasmodium falciparum* major surface protein, was conjugated to PPD and administered in Freund's adjuvant or in saline to BCG primed mice, the anti-$(NANP)_{40}$ antibody titers were equivalent (Lussow et al., 1990b).

Recombinant mycobacterial hsp60 and hsp70 proteins can substitute for PPD in a $(NANP)_{40}$ conjugate (Lussow et al., 1991), suggesting that these were among the components of PPD responsible for the adjuvant-free carrier effect. Hsp60-$(NANP)_{40}$ and hsp70-$(NANP)_{40}$ conjugates were found to elicit anti-$(NANP)_{40}$ antibodies in mice and squirrel monkeys in the absence of adjuvants (Barrios, et al., 1992; Lussow, et al., 1991; Perraut et al., 1993). Moreover, mycobacterial hsp60 and hsp70 were also found to be effective adjuvant-free carriers when conjugated to the poorly immunogenic meningococcal group C oligosaccharide (MenC) (Barrios, et al., 1992).

Other results from the $(NANP)_{40}$ studies support the notion that HSPs can be powerful carriers, but indicate that not all HSPs behave identically. $(NANP)_{40}$ alone acts as a hapten in most strains of mice (Del Giudice et al., 1986; Good et al., 1986), and the presence of covalently linked mycobacterial hsp60 or hsp70 carrier molecules were essential to obtain antibody responses against $(NANP)_{40}$ (Lussow, et al., 1990b). The corresponding heat shock proteins from *E. coli*, GroEL and DnaK, could also function as adjuvant-free carriers to elicit anti-$(NANP)_{40}$ antibodies (Barrios et al., 1994). However, the

hsp70 conjugates exhibited one useful feature that was not observed with hsp60 conjugates. While priming with recombinant hsp60 in Freund's adjuvant or with live BCG was necessary to obtain the carrier effect with the hsp60 conjugates, priming was unnecessary with the hsp70 conjugates. Moreover, previous exposure to BCG or to hsp70 neither augmented nor suppressed the antibody response against the antigen attached to hsp70.

To further investigate the adjuvant-free carrier effect of mycobacterial hsp70, I created an hsp70 fusion vector system (Suzue and Young, 1996). This system enabled the production of proteins composed of one mole of antigen fused to one mole of the hsp70 carrier protein in which the number and position of potential epitopes were identical for each molecule. In contrast, hsp70 conjugates generated by glutaraldehyde cross-linking are a pool of nonidentical molecules with variable epitope density. Immune responses to an antigen can be strongly affected by differences in the molar ratio of antigen and carrier, in the mode of linkage of hapten and carrier and in the position of B and T cell epitopes (Anderson et al., 1989; Dintzis, 1992; Hanna et al., 1972; Klaus and Cross, 1974). Thus, hsp70 fusion proteins reduce the variables associated with the study of HSPs as immunological carriers.

The next chapter of this thesis describes my work in developing the adjuvant-free hsp70 fusion protein system. I used the hsp70 fusion vector system to produce an HIV Gag-hsp70 fusion protein and then investigated the humoral and cellular immune responses elicited against HIV Gag (Suzue and Young, 1996). The mycobacterial hsp70 moiety was found to dramatically increase the immunogenicity of the Gag p24 antigen.

Section II

Chapter 4:
Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24

Summary

Heat shock proteins are major targets of the immune response to bacterial and parasitic pathogens. *Mycobacterium tuberculosis* hsp70 is an especially powerful antigen containing multiple B and T cell epitopes. We investigated whether *M. tuberculosis* hsp70 can be used as an adjuvant-free carrier to stimulate the humoral and cellular immune response to an accompanying protein. A recombinant hsp70 protein expression vector was developed which permits the production of any protein fused to the amino terminus of mycobacterial hsp70. We found that a recombinant HIV p24-hsp70 fusion protein produced with this vector elicited both humoral and cellular immune responses against p24 in mice when administered in the absence of adjuvant. Covalent linkage of hsp70 to p24 was essential to elicit immune responses to p24 in the absence of adjuvant. The anti-p24 IgG_1 antibodies induced in p24-hsp70-immunized mice persisted at high levels for more than one year after immunization. These results demonstrate that the antigenic properties of *M. tuberculosis* hsp70 can be exploited to enhance the humoral and cellular immune response to an attached protein.

Results

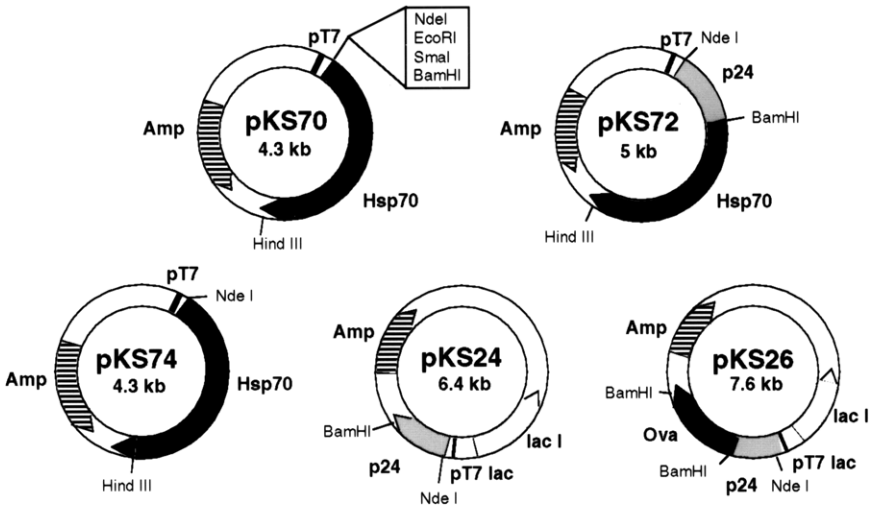
Production and purification of recombinant proteins

A recombinant system was developed to permit production of *M. tuberculosis* hsp70 protein fused to a foreign protein of interest. The vector pKS70 (Fig. 1A) was constructed to permit insertion of DNA between the bacteriophage T7 promoter and the *M. tuberculosis* hsp70 coding sequence, allowing expression of high levels of an hsp70 fusion protein using the T7 expression system (Studier, et al., 1990). Plasmid pKS72 (Fig. 1A), which encodes an HIV-1 p24-hsp70 fusion protein, was then constructed using the pKS70 plasmid. The recombinant fusion protein contained the HIV-1 p24 gag protein at the amino terminus and the hsp70 protein at the carboxyl terminus. Recombinant HIV-1 p24, *M. tuberculosis* hsp70 and p24-ovalbumin proteins were also produced using the related plasmid vectors pKS24, pKS74 and pKS26, respectively (Fig. 1A).

The p24-hsp70 fusion protein, the p24-ovalbumin fusion protein and the hsp70 and HIV-1 p24 proteins were found to be expressed at very high levels in *E. coli* (Fig. 1B; p24-ovalbumin expression not shown). The p24-hsp70 and hsp70 proteins were purified as inclusion bodies and the subsequently refolded proteins were further purified by ATP affinity chromatography and Mono-Q anion exchange chromatography. Histidine tagged HIV-1 p24 and p24-ovalbumin proteins were isolated by using NTA-Ni^{2+} columns. The purity of the recombinant proteins was assessed by SDS-PAGE (Fig. 1B).

Figure 1

A


pT7
NdeI
EcoRI
SmaI
BamHI
Amp
pKS70
4.3 kb
Hsp70
Hind III
pT7
Nde I
p24
BamHI
Amp
pKS72
5 kb
Hsp70
Hind III
pT7
Nde I
Amp
pKS74
4.3 kb
Hsp70
Hind III
Amp
pKS24
6.4 kb
BamHI
lac I
p24
pT7 lac
Nde I
Amp
pKS26
7.6 kb
BamHI
lac I
Ova
BamHI
pT7 lac
p24
Nde I

B

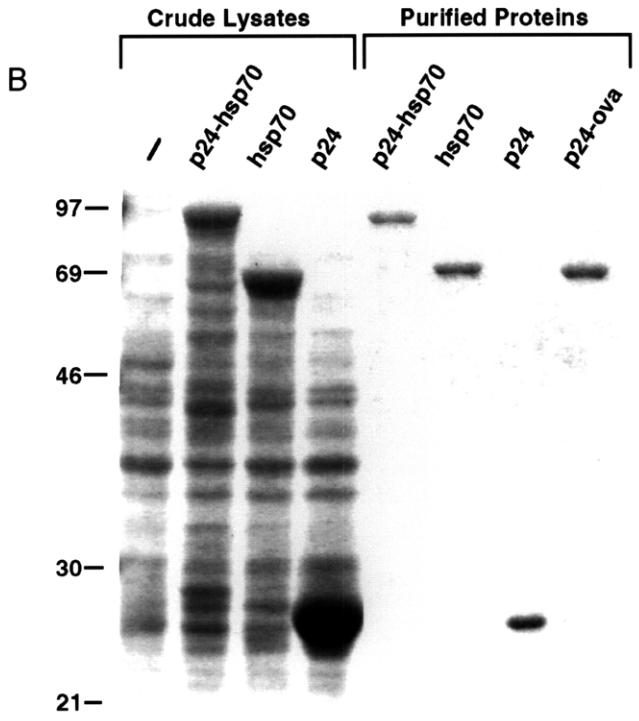

Crude Lysates
Purified Proteins
/
p24-hsp70
hsp70
p24
p24-hsp70
hsp70
p24
p24-ova
97
69
46
30
21

Fig. 1. A. Protein expression vectors. All vectors were constructed for use in the T7 expression system (Studier, et al., 1990) and contain a T7 RNA polymerase promoter. Plasmid pKS70 contains a polylinker that permits introduction of DNA 5′ of the *M. tuberculosis* hsp70 coding sequence. Plasmid pKS72 was constructed for expression of the fusion protein p24-hsp70. Plasmid pKS74 was constructed to express *M. tuberculosis* hsp70 alone (the NdeI site overlaps the AUG initiation codon of hsp70). Plasmid pKS24 can be used to express N-terminal histidine-tagged HIV-1 p24 gag protein. Plasmid pKS26 was constructed for expression of the fusion protein p24-ovalbumin.

B. Production of recombinant proteins. *E. coli* cell lysates and purified proteins were examined by SDS-PAGE and proteins were visualized by Coomassie staining. The gel contains crude extract from *E. coli* containing pKS72 (—), extract from IPTG-induced *E. coli* containing pKS72 (p24-hsp70), extract from IPTG-induced *E. coli* containing pKS74 (hsp70), extract from IPTG-induced *E. coli* containing pKS24 (p24), and the purified proteins p24-hsp70, hsp70, HIV-1 p24 gag and p24-ovalbumin.

E. coli derived recombinant proteins can be contaminated with endotoxins, which have pyrogenic and nonspecific immunostimulatory activities. The endotoxin content of the three purified recombinant proteins was investigated using the *Limulus* amebocyte lysate assay. All of the protein preparations had less than 0.04% endotoxin by weight. Thus, endotoxin contamination of the purified proteins was negligible.

Immunization of mice with p24-hsp70 elicits anti-p24 humoral and cellular responses

We investigated whether mice would elicit an anti-p24 antibody response when injected with the p24-hsp70 fusion protein without adjuvant. Groups of BALB/c mice were inoculated i.p. with various doses of p24-hsp70 fusion protein in PBS. A second equivalent dose was given at three weeks. Serum samples were obtained three weeks after the second immunization, and anti-p24 IgG antibody titers were determined by ELISA. Mice injected with 0.3 μg or 1.2 μg of p24-hsp70 fusion protein had low or undetectable levels of anti-p24 antibody (Fig. 2A). However, mice given doses of 5 μg or 20 μg of p24-hsp70 had high antibody titers, averaging $10^{4.3}$ and $10^{4.2}$ respectively. Thus, administration of two doses of 5 μg of p24-hsp70 fusion protein in the absence of adjuvant is sufficient to induce high levels of anti-p24 IgG antibodies in BALB/c mice.

We examined the ability of the p24-hsp70 fusion protein to elicit anti-p24 cellular responses in the absence of adjuvant. BALB/c mice were immunized as above with various doses of p24-hsp70 and the T cell response to p24 was assessed using *in vitro* cytokine assays. Three weeks after the boost, the splenocytes of immunized mice were removed and cultured *in vitro*. The level of IL-2 secreted by the splenocytes in response to

restimulation with p24 antigen was measured by ELISA. As the immunizing dose of p24-hsp70 increased from 0.3 μg to 5 μg, the amount of IL-2 detected in the splenocyte culture medium also increased (Fig. 2B). The level of *in vitro* IL-2 produced was highest for the 5 μg p24-hsp70 dose group.

Figure 2

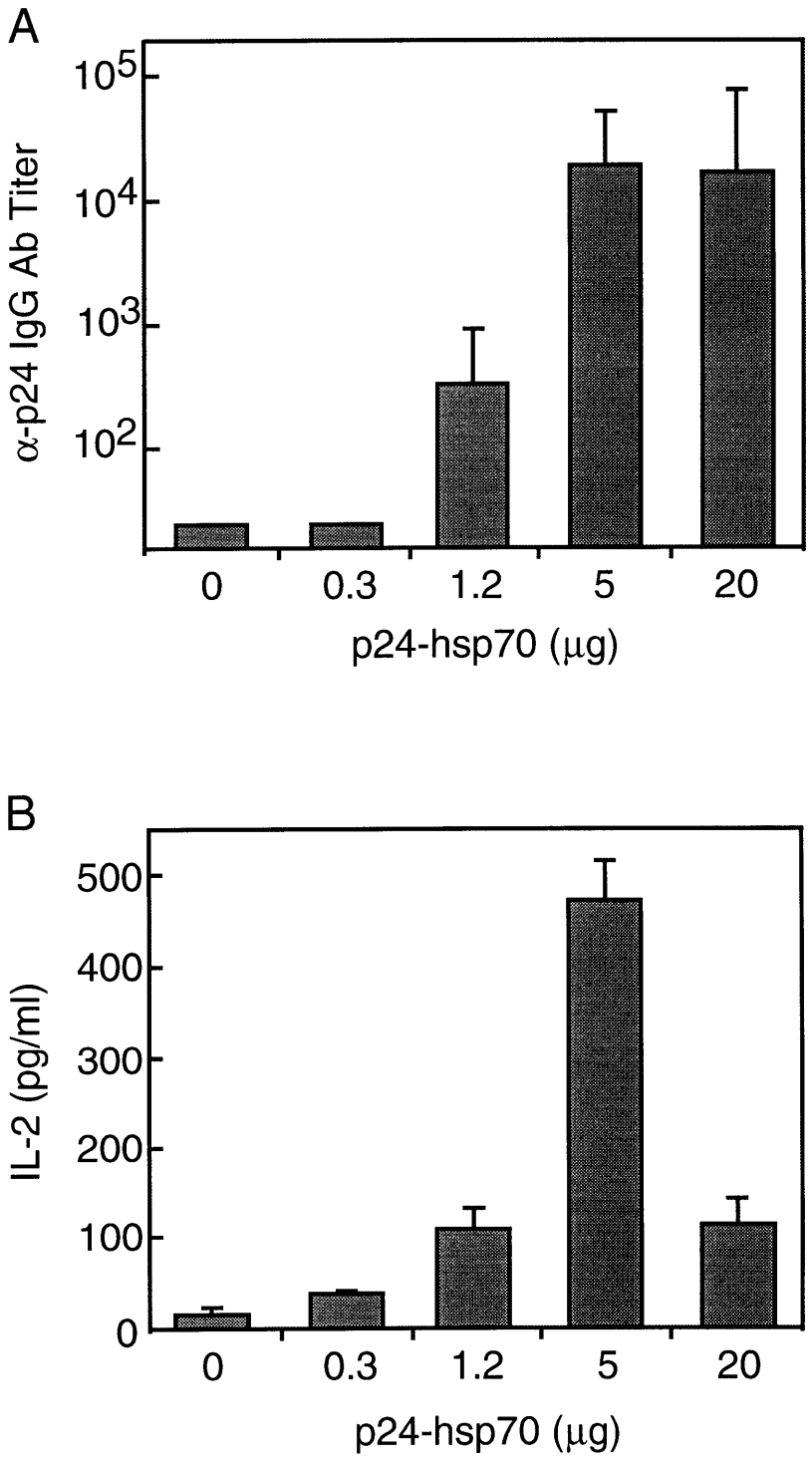

A
α-p24 IgG Ab Titer
10^5
10^4
10^3
10^2
0
0.3
1.2
5
20
p24-hsp70 (μg)
B
IL-2 (pg/ml)
500
400
300
200
100
0
0
0.3
1.2
5
20
p24-hsp70 (μg)

Fig. 2. Immune response of BALB/c mice to various doses of p24-hsp70 fusion protein. Mice were injected i.p. with the indicated amount of fusion protein without adjuvant and boosted 3 weeks later. A) Anti-p24 IgG antibody dose response curve. Mice were bled 3 weeks after the boost and sera was analyzed for anti-p24 IgG antibody by ELISA. The titer is expressed as the highest serum dilution factor giving an absorbance >0.2. The average titer of 4 or 5 mice per group is shown. Titers of $10^{1.4}$ were considered negative for the presence of anti-p24 antibodies. B) IL-2 secretion by splenocytes stimulated *in vitro* with HIV-1 p24 gag antigen (10 μg/ml). For each mouse group, 5 spleens were pooled and processed. Spleens removed 3 weeks or 6 weeks after the boost gave identical results. After 24 hrs of stimulation, culture supernatants were analyzed for IL-2 levels by ELISA. The average of triplicate wells is shown.

Hsp70 is a more effective adjuvant-free carrier protein than ovalbumin

The effectiveness of hsp70 as an adjuvant-free carrier was compared with the carrier protein ovalbumin. Anti-p24 antibody responses were measured in groups of BALB/c mice inoculated with equimolar amounts of p24-hsp70 fusion protein, p24-ovalbumin fusion protein, p24 protein alone or with hsp70 protein alone. Since the immunizing dose of p24-hsp70 that elicited optimal p24-specific antibody and T cell responses was 5 μg (50 pmoles), 50 pmoles of p24-hsp70 and control proteins were used. Mice immunized with p24-ovalbumin had an anti-p24 antibody titer of $10^{2.7}$ (6 weeks after the boost), which was higher than the $10^{1.9}$ antibody titer of mice immunized with p24 alone (Fig. 3A). The highest anti-p24 antibody titer, $10^{4.3}$, was elicited in mice immunized with p24-hsp70 fusion protein.

The p24-specific immune response in each mouse group was further assessed by stimulating spleen cells *in vitro* with p24 antigen. T cell responses were measured using cytokine ELISAs and cell proliferation assays. Splenocytes from mice injected with p24-hsp70 fusion protein released high levels of IFN-γ (10.5 ng/ml) and IL-5 (970 pg/ml) after stimulation *in vitro* with p24 (Fig. 3B & 3C). In contrast, relatively little IFN-γ or IL-5 was produced by splenocytes from mice injected with p24-ovalbumin fusion protein, p24 alone, hsp70 alone or PBS. Levels of IL-4 and IL-10 produced by splenocytes were low (<100 pg/ml and <1 U/ml respectively) in all mouse groups. Results from cell proliferation assays measuring [^{3}H] thymidine incorporation were consistent with the IFN-γ and IL-5 cytokine data in that high levels of p24-specific cell proliferation were elicited only in mice immunized with the p24-hsp70 fusion protein (Fig. 3D).

Figure 3

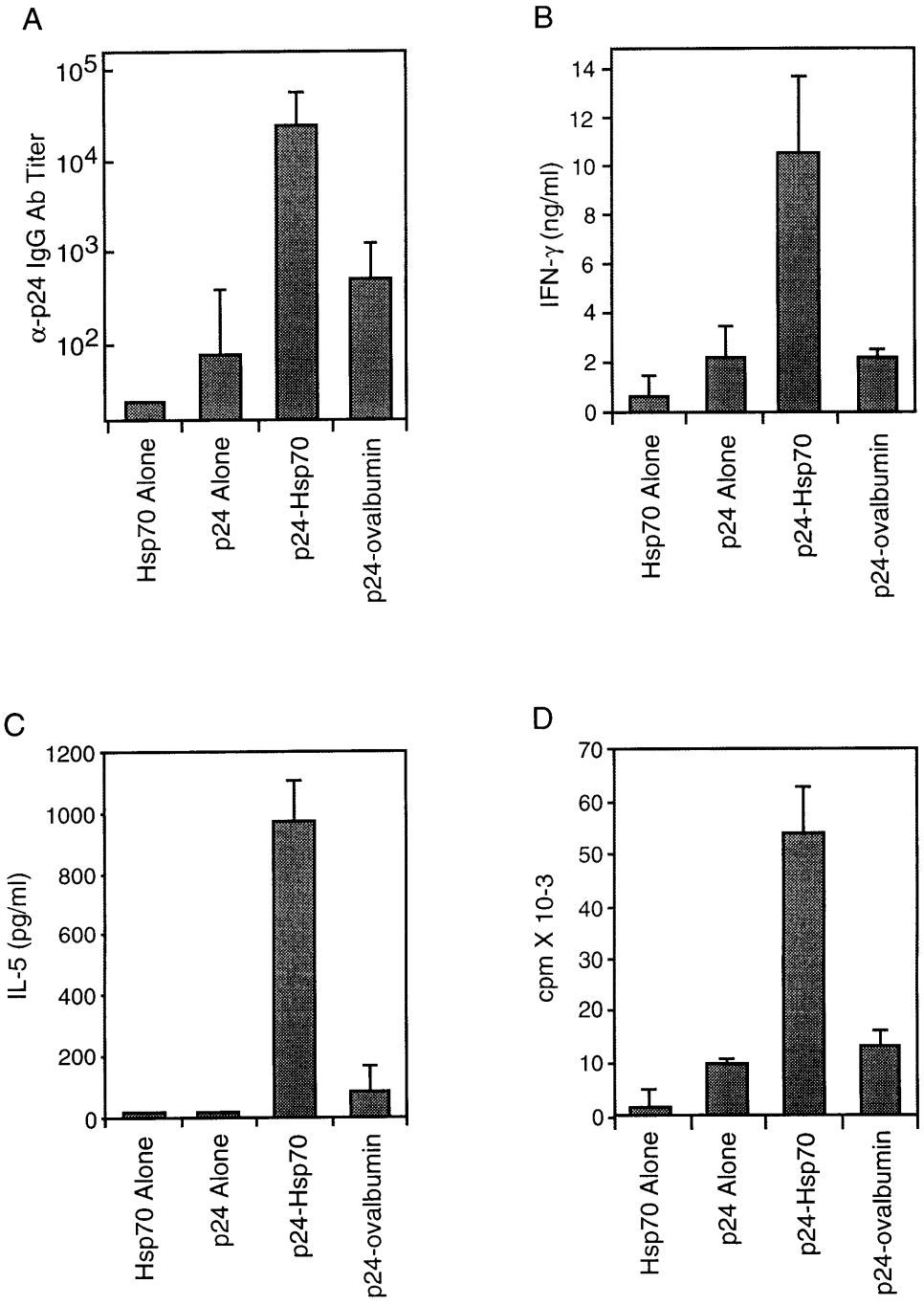

A
α-p24 IgG Ab Titer
10^5
10^4
10^3
10^2
Hsp70 Alone
p24 Alone
p24-Hsp70
p24-ovalbumin
B
IFN-γ (ng/ml)
14
12
10
8
6
4
2
0
Hsp70 Alone
p24 Alone
p24-Hsp70
p24-ovalbumin
C
IL-5 (pg/ml)
1200
1000
800
600
400
200
0
Hsp70 Alone
p24 Alone
p24-Hsp70
p24-ovalbumin
D
cpm X 10-3
70
60
50
40
30
20
10
0
Hsp70 Alone
p24 Alone
p24-Hsp70
p24-ovalbumin

Fig. 3. Comparison of hsp70 and ovalbumin as carrier proteins. Mice were injected i.p. with 50 pmoles of one of the following proteins: p24-hsp70, p24-ovalbumin, p24 or hsp70. Mice were boosted 3 weeks later A) Anti-p24 IgG antibody titers 6 weeks after the boost, determined as described in the Figure 2 legend. B-D) Cellular response of BALB/c mice inoculated with recombinant proteins in the absence of adjuvant. Mice were sacrificed 6 weeks after the boost and for each mouse group, 5 spleens were pooled and processed. B) IFN-γ secretion and C) IL-5 secretion by splenocytes stimulated *in vitro* with p24 as measured by ELISA. All samples were done in triplicate. D) Cell proliferation. Splenocytes were stimulated *in vitro* with p24 antigen and pulsed on day 3 with [^{3}H] thymidine. The average Δcpm value from two experiments is shown.

Hsp70 must be covalently coupled to p24 to engender immune responses

We investigated whether the carrier effect of hsp70 depends on physical linkage of hsp70 to p24. It is possible that hsp70 acts as a classic adjuvant, in which case it should stimulate the immune response to an antigen when mixed with, but not covalently coupled to, the antigen. Mice were injected with 50 pmoles of p24-hsp70 fusion protein or with 50 pmoles of hsp70 mixed with but not attached to, 50 pmoles of p24. Both anti-p24 antibody titers and IL-2 production by splenocytes were measured. The presence of anti-p24 antibody was barely detectable in mice injected with the hsp70, p24 protein mix (Fig. 4A). In comparison, the mice injected with the p24-hsp70 fusion protein had at least 1000 times greater anti-p24 antibody levels. Similar results were obtained in splenocyte assays; very low levels of IL-2 were produced by splenocytes of mice injected with the hsp70, p24 protein mix whereas high levels of IL-2 were secreted by the splenocytes of the fusion protein mouse group (Fig. 4B). These experiments indicate that hsp70 engenders substantial immune responses to p24 only when physically attached to p24.

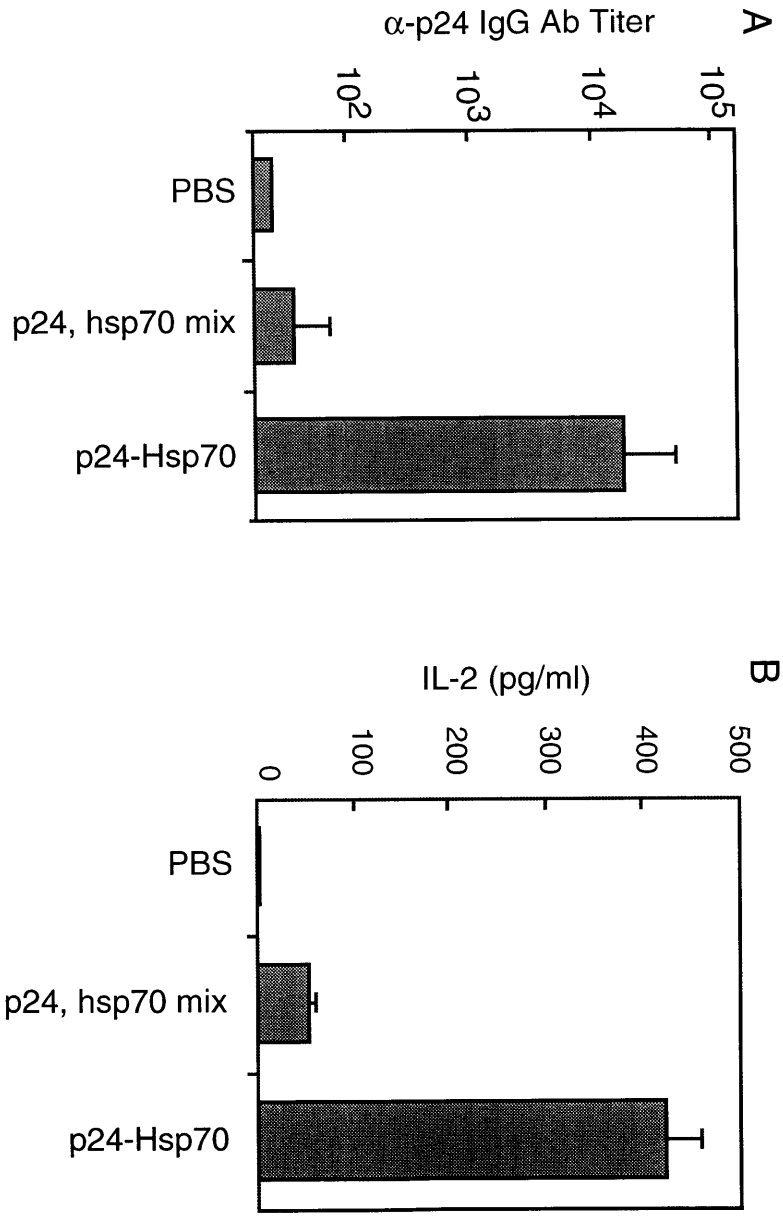

A
α-p24 IgG Ab Titer
10^2
10^3
10^4
10^5
PBS
p24, hsp70 mix
p24-Hsp70
B
IL-2 (pg/ml)
0
100
200
300
400
500
PBS
p24, hsp70 mix
p24-Hsp70

Fig. 4. Immune response of mice injected with p24-hsp70 fusion protein or with a mixture of p24 and hsp70. Mice were injected i.p. with 50 pmoles of the fusion protein or with 50 pmoles each of p24 and hsp70. A) Anti-p24 IgG antibody titers, determined as described in the legend to Figure 2. B) IL-2 secretion by splenocytes stimulated *in vitro* with HIV-1 p24 gag antigen, determined as described in the legend to Figure 2.

Adjuvant-free immunization of mice with p24-hsp70 elicits long-lived anti-p24 IgG_1 antibody response

The anti-p24 antibody response was monitored over a period of 71 weeks in mice immunized with p24-hsp70 fusion protein, p24, hsp70 or PBS. None of the immunogens included an adjuvant. Since 5 μg (50 pmole) of p24-hsp70 was optimal for inducing antibody responses in previous experiments, 50 pmole doses of each of the proteins were used in the time course experiment. Figure 5A shows that the antibody response elicited by the p24-hsp70 fusion protein was long-lasting; 36 weeks after the boost, the average anti-p24 antibody titer was above 10^4 and 68 weeks after boost, the titer was $10^{3.6}$. Some mice injected with p24 protein alone had low but detectable anti-p24 antibody titers soon after the boost, but anti-p24 antibodies in these mice were not long-lasting. As expected, there was no detectable anti-p24 antibody in mice immunized with hsp70 or PBS.

In mice immunized with p24-hsp70 fusion protein, the anti-p24 IgG antibodies detected 9 days after the boost were IgG_1, IgG_{2a} and IgG_{2b} isotype antibodies (Fig. 5B). Anti-p24 IgG_3 and IgA antibodies were not detected. The anti-p24 antibodies 9 days after the boost were approximately >90% IgG_1. The levels of anti-p24 IgG_{2a} and IgG_{2b} antibodies 6 weeks after the boost were significantly lower than at 9 days after the boost (Fig. 5B). In contrast, the anti-p24 IgG_1 antibody response was long-lasting and the levels detected 9 days after the boost and 36 weeks after the boost were nearly identical.

Figure 5

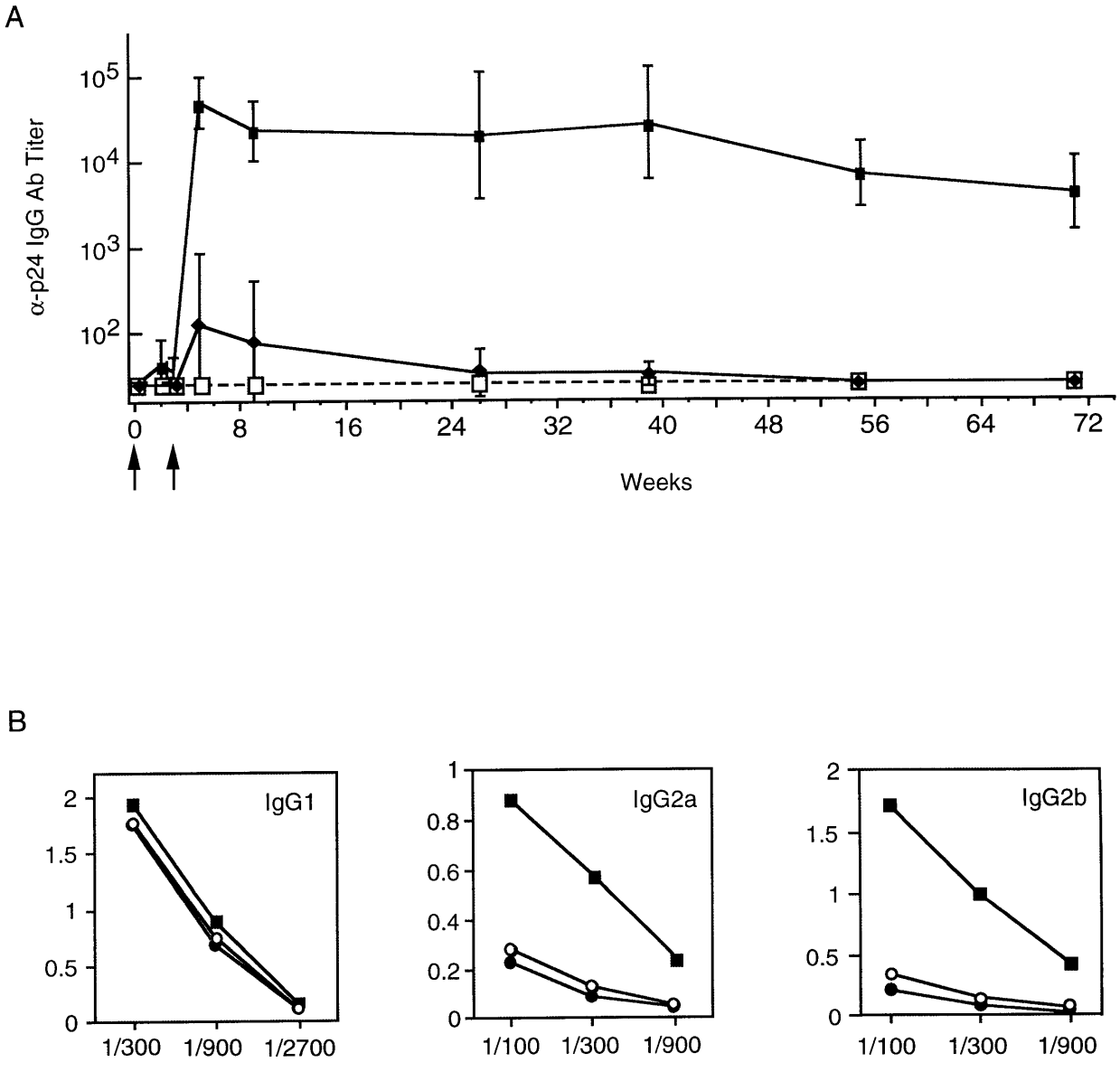

A
α-p24 IgG Ab Titer
10^5
10^4
10^3
10^2
0
8
16
24
32
40
48
56
64
72
Weeks
B
IgG1
2
1.5
1
0.5
0
1/300
1/900
1/2700
IgG2a
1
0.8
0.6
0.4
0.2
0
1/100
1/300
1/900
IgG2b
2
1.5
1
0.5
0
1/100
1/300
1/900

Fig. 5. A. Time course of the anti-p24 IgG antibody response. Mice were injected on day 1 and 21 (as indicated, ↑) with 50 pmoles of either p24-hsp70 fusion protein —■—, p24 —◆—, or with hsp70 —□—. Mice were bled at the indicated time points and the serum samples were tested for anti-p24 IgG antibody levels by ELISA. Results obtained with mice injected with PBS or with hsp70 protein were identical. The average titer for 4 to 5 mice per group is shown. Titer is expressed as the highest serum dilution factor positive in the ELISA.

B. Isotype analysis of anti-p24 antibodies. Serum samples from mice immunized with 50 pmoles of p24-hsp70 were analyzed for anti-p24 IgG_1, IgG_{2a} and IgG_{2b} antibodies on the following days after the boost: 9 days —■—, 6 weeks —○— and 36 weeks —●—. Results are expressed as 450 nm absorbance values.

Discussion

The hsp70 fusion vector described here enabled the expression of recombinant HIV p24-hsp70 fusion protein. Fusing mycobacterial hsp70 to HIV-1 gag p24 enhanced the immunogenicity of the p24 antigen and obviated the need for an exogenous adjuvant. Both humoral and cellular immune responses against p24 could be elicited by administering p24-hsp70 fusion protein in phosphate buffer. In order for hsp70 to augment anti-p24 immune responses in the absence of adjuvant, physical linkage of hsp70 to p24 was essential.

In this study, recombinant p24-hsp70 fusion protein was produced for use as an immunogen using a vector system that permits the production of any hsp70 fusion protein. In principle, any gene or epitope of interest can be subcloned into the plasmid vector pKS70 and antigen fused to hsp70 can be expressed and purified. For studies of the adjuvant-free carrier effect of hsp70, these fusion proteins have several advantages. The hsp70 fusion proteins are easy to produce in large amounts, to purify and to characterize. This vector system enables the production of proteins composed of one mole of antigen fused to one mole of the hsp70 carrier protein in which the number and position of potential epitopes were identical for each molecule. In contrast, hsp70 conjugates generated by glutaraldehyde cross-linking (Barrios, et al., 1992; Lussow, et al., 1991; Perraut, et al., 1993) are a pool of nonidentical molecules with variable epitope density. Immune responses to an antigen can be strongly affected by differences in the molar ratio of antigen and carrier, in the mode of linkage of hapten and carrier and in the position of B and T cell epitopes (Anderson, et al., 1989; Cox et al., 1988; Dintzis, 1992; Hanna, et al., 1972; Klaus and Cross, 1974; Lowenadler and Lycke, 1994; Lowenadler et al.,

1992; Snippe et al., 1975). Thus, hsp70 fusion proteins reduce the variables associated with the study of HSPs as immunological carriers.

Inoculation of mice with the hsp70-p24 fusion protein elicited a strong antibody response to HIV-1 p24 when administered in the absence of adjuvant. These results show that the adjuvant-free carrier effect of hsp70, previously observed when cross-linked to high molar amounts of synthetic peptide or oligosaccharide (Barrios, et al., 1992; Lussow, et al., 1991; Perraut, et al., 1993), can be extended to an hsp70 fusion protein where the antigen is a full-length protein, and where the immunogen contains equivalent molar amounts of hsp70 and the antigen of interest. Thus, our studies demonstrate the effectiveness of hsp70 as an adjuvant-free carrier when used with low epitope density of antigen.

The antibody response elicited by the hsp70 fusion protein was surprisingly long-lived. Mice immunized with p24-hsp70 fusion protein maintained high levels of anti-p24 antibody for at least 68 weeks after immunization. The antibody isotypes secreted *in vivo* are regulated by cytokines, with TH1 cytokines preferentially eliciting IgG_{2a} and TH2 cytokines stimulating IgG_1 (Coffman et al., 1988; Finkelman et al., 1990). In p24-hsp70 immunized mice, anti-p24 IgG_1, IgG_{2a} and IgG_{2b} antibodies were elcited and in particular, the anti-p24 IgG_1 antibodies persisted at high levels.

The experiments described here also demonstrated that the adjuvant-free carrier effect of hsp70 extends to the stimulation of cellular responses to a foreign antigen. Splenocytes from the fusion protein-immunized mice exhibited p24 antigen-dependent proliferation and production of IL-2, IL-5 and IFN-γ. On the basis of cytokine secretion patterns, murine helper T cell clones have been categorized as TH1 or TH2 (Mosmann and Coffman, 1989). Immunization of mice with p24-hsp70 elicited p24 specific TH1 and TH2 cells

since both TH1 (IL-2 and IFN-γ) and TH2 (IL5) type cytokines were secreted in response to p24 stimulation *in vitro*.

The powerful carrier effect of hsp70 was also evident in comparative studies with the carrier protein ovalbumin. The mycobacterial hsp70 moiety was found to dramatically increase the immunogenicity of the HIV-1 gag p24 antigen in the absence of adjuvant. In contrast, mice immunized with the p24-ovalbumin fusion protein failed to elicit high levels of anti-p24 immune responses.

Covalent linkage of hsp70 to p24 was essential to elicit humoral and cellular immune responses to p24 in the absence of adjuvant under the conditions used in these experiments. These results are consistent with the recent report that an anti-$(NANP)_{40}$ antibody response is elicited in mice when hsp70 is cross-linked but not when it is simply mixed with the malarial peptide $(NANP)_{40}$ (Barrios, et al., 1994). Since $(NANP)_{40}$ acts as a hapten in these mice strains (Del Giudice, et al., 1986; Good, et al., 1986), $(NANP)_{40}$ administered with Freund's adjuvant is also ineffective in eliciting anti-$(NANP)_{40}$ antibodies. Thus, it was not clear from previous studies whether the adjuvant-free immunostimulatory effect of hsp70 was directly related to its carrier function of supplying T cell epitopes. By using the non-haptenic antigen HIV p24, we found that, unlike conventional adjuvants, hsp70 could not be merely mixed with the antigen. Physical linkage of hsp70 to the non-haptenic antigen p24 was necessary in order for hsp70 to exert an adjuvant-free carrier effect with p24.

This requisite physical attachment of hsp70 to the p24 antigen implies that p24, which already contains numerous T cell epitopes, becomes more immunogenic due to the addition of hsp70 T cell epitopes. It has been suggested that a high precursor frequency of high affinity hsp70 reactive T

cells exists due to the continual exposure of the immune system to hsp70 from commensal or pathogenic organisms (Kaufmann and Schoel, 1994b; Murray and Young, 1992). Immune responses to hsp70 have been detected following exposure to a broad spectrum of infectious agents (Anzola et al., 1992; Hedstrom et al., 1987; Rothstein et al., 1989; Selkirk et al., 1989; Young et al., 1988). In addition, anti-hsp70 immune responses were induced in infants by the trivalent vaccine against tetanus, diphtheria and pertussis (Del Giudice et al., 1993). It seems that the immune system is routinely stimulated to respond to hsp70 and such stimulation may cause an expansion of hsp70 reactive T cells.

How does hsp70 exert its adjuvant-free carrier effect? We suggest that the humoral response against p24 is augmented by hsp70 in the following manner. When animals are primed with p24-hsp70 fusion protein, and then exposed to a second dose, a p24-specific B cell recognizes, internalizes and processes the p24-hsp70 fusion protein. The B cell then efficiently presents peptides derived from hsp70 on its surface in the context of an MHC molecule. This MHC/hsp70 peptide complex is bound by a primed, hsp70-reactive T cell, leading to the directed release of cytokines by the T cell to the B cell. These soluble factors stimulate the B cell to proliferate, differentiate and secrete anti-p24 antibodies.

Other factors may contribute to the mechanism by which hsp70 exerts its adjuvant-free carrier effect. Hsp70 has been implicated in antigen presentation (De Nagel and Pierce, 1992; Vanbuskirk et al., 1989) and it is possible that this function of hsp70 contributes to its adjuvant-free carrier effect. It may also be that relative to T cell epitopes of other proteins, hsp70 T cell epitopes are processed by antigen presenting cells more efficiently and that the resulting hsp70 peptides bind with high affinity to MHC molecules.

Further experiments must be performed to address the adjuvant-free carrier mechanism of hsp70.

The lack of optimal adjuvants and carrier proteins have been problematic in vaccine development (Altman and Dixon, 1989; Del Giudice, 1992) and the use of hsp70 may be a practical alternative. The present study illustrates the effectiveness of hsp70 in eliciting a humoral and cellular response to an attached molecule in the absence of adjuvant and affirms the potential utility of hsp70 in vaccine development.

Materials and Methods

Expression Vector Constructs

The DNA fragment containing the *M. tuberculosis* hsp70 coding sequence was synthesized by PCR using DNA purified from λgt11 clones Y3111 and Y3130 (Young et al., 1987) as a template. One upstream primer (5'CCCGG**CATATG**GCTCGTGCGGTCGG3') contained an *NdeI* site that overlapped the AUG translation initiation codon of the hsp70 gene and another upstream primer (5'GCCCG**GGATCC**ATGGCTCGTGCGGTCGGGAT3') contained a *BamHI* site immediately before the hsp70 coding sequence. The downstream primer (5'GGACAC**AAGCTT**TCATCAGCCGAGCCG3') contained a *HindIII* site immediately after the translation stop codon.

The DNA fragment containing the HIV-1 gag p24 coding sequence was synthesized by PCR using plasmid pHXB2 (Fisher et al., 1985) as a template. The upstream primer (5'CCTCGTG**CATATG**CCTATAGTGCAGAACATCCAGGG3') contained an *NdeI* site that overlapped the AUG translation initiation codon for the p24 gene. The downstream primers contained a *BamHI* site either immediately after the last coding sequence or immediately after the translation stop codon (5'CG**GGATCC**CAAAACTCTTGCCTTATGGCC3'; 5'CG**GGATCC**TCATTACAAAACTCTTGCCTTATG3').

The DNA fragment containing the ovalbumin coding sequence was synthesized by PCR using plasmid pOv230 (McReynolds et al., 1978; McReynolds et al., 1977) as a template. The upstream primer contained a *BamHI* site immediately before the ovalbumin coding sequence (5'GTC**GGATCC**ATGGGCTCCATCGGCGCAGCA3') and the downstream

primer contained a *BamHI* site immediately after the translation stop codon (5'GCA**GGATCC**TTAAGGGGAAACACATCTG3').

The plasmid vector pKS70 was created by subcloning the *M. tuberculosis* hsp70 gene into the expression vector pT7-7 (Tabor and Richardson, 1985) at the *BamHI* and *HindIII* sites (Fig. 1A). DNA encoding HIV-1 p24 was then subcloned into pKS70 at the *NdeI* and *BamHI* sites to generate pKS72 (Fig 1A). Plasmid pKS74 was created by subcloning hsp70 into the *NdeI* and *HindIII* sites of pT7-7 (Fig 1A). The expression vector pET11 (Studier et al., 1990) was modified with a histidine tag coding sequence and DNA encoding HIV-1 p24 was subcloned at the *NdeI* and *BamHI* sites, producing pKS24 (Fig 1A). Plasmid pKS26 was created by subcloning ovalbumin into the *BamHI* site of pKS24. All plasmids were propagated in *E. coli* DH5α. Recombinant protein expression was carried out in *E. coli* BL21(DE3)pLysS (Studier, et al., 1990).

Protein Purification

Overnight cultures of BL21(DE3)pLysS were diluted 1/100 in 2XYT medium containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml). The cultures were grown to an OD_{600} of 0.5 and recombinant protein production was induced by addition of IPTG (0.5 mM) to the culture. After 30 min., rifampicin (150 μg/ml) was added to cells expressing recombinant hsp70 and to cells expressing p24-hsp70. All cultures were induced with IPTG for a total of 4 hrs. The cells were harvested and cell pellets were frozen in ethanol/dry ice.

Hsp70 and p24-hsp70 proteins both formed inclusion bodies and were purified as follows. To prepare inclusion bodies, cell pellets were resuspended in ice-cold lysis buffer (25 mM HEPES pH 7.3, 12.5 mM $MgCl_2$, 0.1

mM $ZnCl_2$, 0.1% NP-40, 20% glycerol, 1.25 M LiCl, 1 mM PMSF). The cells were sonicated and then pelleted by centrifugation at 15,000 rpm for 10 min. in a Sorvall SS-34 rotor. The pellet was resuspended in buffer A (20 mM Tris pH 8, 20 mM EDTA, 0.5 mg/ml lysozyme), the suspended cells were sonicated, placed on ice for 30 min. and pelleted by centrifugation at 15,000 rpm for 10 min. The pellet was resuspended in buffer B (10 mM Tris pH 8, 1 mM EDTA, 1.25 M LiCl, 0.5% NP-40), sonicated and centrifuged at 15,000 rpm for 15 min. After repeating the buffer B wash, the pellet was resuspended in buffer C (10 mM Tris pH 8, 1 mM EDTA, 0.5% NP-40), sonicated and centrifuged at 15,000 rpm for 15 min. The buffer C wash was repeated.

The hsp70 and p24-hsp70 inclusion bodies were each solubilized in buffer D (10 mM Tris pH 8, 20 mM EDTA, 0.1 M KCl) containing 5 M guanidine. Proteins were refolded stepwise by dialyzing against buffer D plus 2 M guanidine HCl, buffer D plus 1 M guanidine, buffer D plus 0.5 M guanidine, buffer D plus 0.25 M guanidine and finally against HEPES buffer (25 mM HEPES pH 7.3, 12.5 mM $MgCl_2$, 20 mM EDTA).

For ATP affinity chromatography, ATP-agarose (Sigma) was equilibrated in buffer E (100 mM Tris pH 7.5, 4 mM $MgCl_2$) plus 0.2% NP-40 and 100 mM NaCl. Protein was loaded on the ATP-column and subsequently the column was rinsed with buffer E plus 0.2% NP-40 and 600 mM NaCl. The column was then rinsed with buffer E plus 100 mM NaCl and then with buffer E containing 100 mM NaCl and 5 mM ATP. Hsp70 and p24-hsp70 were eluted with buffer E plus 100 mM NaCl and 25 mM ATP. An aliquot of each fraction was analyzed by SDS-PAGE and pooled fractions were dialyzed against buffer F (20 mM Tris pH 8, 1 mM $MgCl_2$).

Hsp70 or p24-hsp70 protein was loaded on a FPLC Mono-Q HR 5/5 anion exchange column (Pharmacia) equilibrated in buffer F. After washing

with buffer F plus 50 mM NaCl, protein was eluted with a 50 mM to 500 mM gradient of sodium chloride in buffer F. After examination by SDS-PAGE, fractions containing the most pure protein were pooled and dialyzed against PBS.

The HIV-1 p24 gag protein and the p24-ovalbumin fusion protein were expressed in *E. coli* as described above. Cells were resuspended in lysis buffer (1% NP-40, 10 mM 2-ME, 1 mM PMSF, 5% glycerol, 50 mM pH 8 sodium phosphate, 300 mM NaCl, 30 mM imidazole) and sonicated. The lysate was then centrifuged at 15,000 rpm for 10 min. in a Sorvall SS-34 rotor. The clarified cell lysate was loaded on a nitrilo-tri-acetic acid (NTA) Ni^{2+} column (Qiagen) and the column was then rinsed with 20 column volumes of lysis buffer. For purification of p24-ova fusion protein, the column was further rinsed with an additional ten column volumes of buffer Z (300 mM NaCl, 50 mM pH 6 sodium phosphate) plus 60 mM imidazole and the p24-ova protein was eluted with buffer Z plus 100 mM imidazole. In the HIV-1 p24 gag protein purification, buffer Z plus 100 mM imidazole was used for rinsing the column and the p24 protein was eluted with buffer Z plus 500 mM imidazole. An aliquot of each 1.5 ml fraction was examined by SDS-PAGE. Pooled fractions were dialyzed against PBS. The purified p24, hsp70, p24-ovalbumin and p24-hsp70 proteins were stored at -70°C.

Protein Analysis

Sample aliquots were resuspended in Laemmli buffer (Laemmli, 1970) and subjected to SDS-PAGE. Protein was visualized by Coomassie staining. For Western blotting, proteins were transferred from the gel to a Bio-blot nitrocellulose membrane (Costar) and probed with antibodies. The anti-mycobacterial hsp70 mAb IT-41 was obtained from the World Health

Organization Mycobacterial Monoclonal Antibody Bank (Dr. T. Shinnick, Atlanta, GA), the anti-DnaK mAb was from Dr. L. Mizzen (StressGen, Victoria, B.C.), the rabbit anti-p24 (HIV-1) antibody was from Intracel (Cambridge, MA) and the rabbit anti-ovalbumin antibody was from Sigma.

Protein concentrations were determined by the bicinchoninic acid assay (Pierce). Purified proteins were analyzed for endotoxin content using the *Limulus* amebocyte lysate assay (Sigma).

Mice and Immunizations

7-8 week old female BALB/c mice were obtained from Taconic Farms (Germantown, NY). Mice were immunized i.p. on day 0 and day 21 with doses ranging up to 20 μg of purified protein in PBS. Doses of 20 μg, 5 μg (50 pmoles), 1.2 μg and 0.3 μg of p24-hsp70 fusion protein were used. When mice were immunized with hsp70 alone, HIV-1 p24 alone or with a mixture of hsp70 and HIV-1 p24, 3.8 μg (50 pmoles) hsp70 and 1.2 μg (50 pmoles) HIV-1 p24 were used. When mice were injected with p24-ovalbumin fusion protein, 3.5 μg (50 pmoles) was used. Mice were periodically bled from the retroorbital plexus.

Determination of Antibody Titers and Isotypes

A 96 well flat bottom ELISA plate was coated overnight at room temperature with 50 μl of p24 (2.5 μg/ml). The plate was rinsed with PBS, incubated with blocking buffer (5% nonfat dry milk powder and 0.2% Tween-20 in PBS) for 2 hrs at 37°C, and again rinsed with PBS. Mouse serum samples were diluted in blocking buffer, added to the plate and incubated for 2 hrs at 37°C. After rinsing with PBS, the plate was incubated with HRP conjugated anti-mouse IgG (Pierce; Pharmingen) for 1 hr at 37°C. After extensive

washing, 3,3',5,5' tetramethylbenzidine (TMB) substrate was added. After 20 min. at RT, the reaction was stopped with 2 M H_2SO_4 and absorbance was read at 450 nm. The titer is expressed as the highest serum dilution factor giving an absorbance >0.2.

Isotype specific analyses were done by ELISA using one of the following HRP conjugated anti-mouse Ig: anti-IgG_1, anti-IgG_{2a}, anti-IgG_{2b}, anti-IgG_3 or anti-IgA (American Qualex, La Mirada, CA; Pierce, Pharmingen). Due to the inherent difficulty of designing accurate standards for anti-p24 antibodies of these various isotypes, the ELISA results are expressed as 450 nm absorbance values.

Cell Proliferation Assay

Spleens were removed from mice 3-6 weeks after the last injection. The spleens from 5-10 mice in each treatment group were pooled. Single-cell suspensions were prepared by grinding tissue through a sterile nylon mesh. Splenocytes were purified by Ficoll-Paque (Pharmacia) density centrifugation. 3-4 X 10^6 cells/ml were cultured in RPMI 1640:DMEM (1:1), supplemented with 10% FCS and 50 μM 2-ME at 37°C in 96-well flat bottom microculture plates in 5% CO^2. The cells were stimulated with 10 μg/ml p24 (triplicate samples). On day 3 of culture, [^{3}H] thymidine (1 μCi/well) was added for 16 hrs. The cells were harvested and [^{3}H] thymidine incorporation was measured in a scintillation counter. Results are expressed as Δcpm (Δcpm = arithmetic mean of cpm from p24 stimulated cultures — arithmetic mean of cpm from corresponding control cultures).

Cytokine ELISAs

Splenocytes were prepared as above and cultured in 96-well round bottom microculture plates. Cells were stimulated with p24 (10 μg/ml), Con A (5 μg/ml) or with HIV-1 gag peptides (10 μg/ml of ea. peptide). The peptides used were 25 amino acids each, contained 8 overlapping amino acid residues and encompassed the HIV-1 gag p55 residues 256-348 (Aldovini and Young, 1991). Cell culture supernatants were removed at 48 hrs to assay for IL-2 and at 72 hrs for all other cytokines. A sandwich ELISA using paired monoclonal antibodies (Pharmingen or Endogen) was used to measure IL-2, IL-4, IL-5 and IFN-γ and an Endogen ELISA kit was used to measure IL-10.

Acknowledgments

We thank Drs. Peter Murray and Anna Aldovini for valuable suggestions and stimulating discussions. We also thank Dr. Jerry Nau for critical reading of the manuscript.

Footnote

[1]This work was supported by National Institutes of Health Grants AI23545 and AI26463 and by a predoctoral fellowship from the Office of Naval Research.

Section II References

Aldovini, A. and R. A. Young. 1991. Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines. *Nature* 351:479-82.

Altman, A. and F. J. Dixon. 1989. Immunomodifiers in Vaccines. In *Advances in Veterinary Science and Comparative Medicine*, J. L. Bittle and F. L. Murphy, eds. Academic Press, San Diego, p. 313-316.

Anderson, P. W., M. E. Pichichero, E. C. Stein, S. Porcelli, R. F. Betts, D. M. Connuck, D. Korones, R. A. Insel, J. M. Zahradnik and R. Eby. 1989. Effect of oligosaccharide chain length, exposed terminal group, and hapten loading on the antibody response of human adults and infants to vaccines consisting of Haemophilus influenzae type b capsular antigen unterminally coupled to the diphtheria protein CRM197. *J Immunol* 142:2464-8.

Anzola, J., B. J. Luft, G. Gorgone, R. J. Dattwyler, C. Soderberg, R. Lahesmaa and G. Peltz. 1992. Borrelia burgdorferi HSP70 homolog: characterization of an immunoreactive stress protein. *Infect Immun* 60:3704-13.

Audibert, F. M. and L. D. Lise. 1993. Adjuvants: current status, clinical perspectives and future prospects. *Immunol Today* 14:281-4.

Barrios, C., C. Georgopoulos, P. H. Lambert and G. Del Giudice. 1994. Heat shock proteins as carrier molecules: in vivo helper effect mediated by Escherichia coli GroEL and DnaK proteins requires cross-linking with antigen. *Clin Exp Immunol* 98:229-33.

Barrios, C., A. R. Lussow, J. Van Embden, R. Van der Zee, R. Rappuoli, P. Costantino, J. A. Louis, P. H. Lambert and G. Del Giudice. 1992. Mycobacterial heat-shock proteins as carrier molecules. II: The use of the 70-kDa mycobacterial heat-shock protein as carrier for conjugated vaccines can circumvent the need for adjuvants and Bacillus Calmette Guerin priming. *Eur J Immunol* 22:1365-72.

Claman, H. N., E. A. Chaperon and R. F. Triplett. 1966. Thymus-marrow cell combinations. Synergism in antibody production. *Proc Soc Exp Biol Med* 122:1167-71.

Coffman, R. L., B. W. P. Seymour, D. A. Lebman, D. D. Hiraki, J. A. Christiansen, B. Shrader, H. M. Cherwinski, H. F. J. Savelkoul, F. D. Finkelman, M. W. Bond and T. R. Mosmann. 1988. The role of helper T cell products in mouse B cell differentiation and isotype regulation. *Immunological Reviews* 102:5-28.

Cox, J. C. and A. R. Coulter. 1997. Adjuvants-a classification and review of their modes of action. *Vaccine* 15:248-256.

Cox, J. H., J. Ivanyi, D. B. Young, J. R. Lamb, A. D. Syred and M. J. Francis. 1988. Orientation of epitopes influences the immunogenicity of synthetic peptide dimers. *Eur J Immunol* 18:2015-9.

Davenport, F. M., A. V. Hennessy and F. B. Askin. 1968. Lack of adjuvant effect of Alum on purified influenza virus hemagglutinins in man. *J Immunol* 100:1139-1140.

Davies, A. J. S., E. Leuchars, V. Wallis, R. Marchant and E. V. Elliott. 1967. The failure of thymus-derived cells to produce antibody. *Transplantation* 5:222-231.

De Nagel, D. C. and S. K. Pierce. 1992. A case for chaperones in antigen processing. *Immunol Today* 13:86-9.

Del Giudice, G. 1992. New carriers and adjuvants in the development of vaccines. *Curr Opin Immunol* 4:454-9.

Del Giudice, G., J. A. Cooper, J. Merino, A. S. Verdini, A. Pessi, A. R. Togna, H. D. Engers, G. Corradin and P. H. Lambert. 1986. The antibody response in mice to carrier-free synthetic polymers of Plasmodium falciparum circumsporozoite repetitive epitope is I-Ab-restricted: possible implications for malaria vaccines. *J Immunol* 137:2952-5.

Del Giudice, G., A. Gervaix, P. Costantino, C. A. Wyler, C. Tougne, E. R. de Graeff-Meeder, J. Van Embden, R. Van der Zee, L. Nencioni, R. Rappuoli, S. Suter and P. H. Lambert. 1993. Priming to heat shock proteins in infants vaccinated against pertussis. *J Immunol* 150:2025-32.

Dintzis, R. Z. 1992. Rational design of conjugate vaccines. *Pediatr Res* 32:376-85.

Edelman, R. and C. O. Tacket. 1990. Adjuvants. *Int Rev Immunol* 7:51-66.

Finkelman, F. D., J. Holmes, I. M. Katona, J. F. Urban, M. P. Beckmann, L. S. Park, K. A. Schooley, R. L. Coffman, T. R. Mosmann and W. E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303-33.

Fisher, A. G., E. Collalti, L. Ratner, R. C. Gallo and F. Wong-Staal. 1985. A molecular clone of HTLV-III with biological activity. *Nature* 316:262-5.

Geerligs, H. J., W. J. Weijer, G. W. Welling and S. Welling-Wester. 1989. The influence of different adjuvants on the immune response to a synthetic peptide comprising amino acid residues 9-21 of herpes simplex virus type 1 glycoprotein D. *J Immunol Methods* 124:95-102.

Good, M. F., J. A. Berzofsky, W. L. Maloy, Y. Hayashi, N. Fujii, W. T. Hockmeyer and L. H. Miller. 1986. Genetic control of the immune response in mice to a Plasmodium falciparum sporozoite vaccine. Widespread nonresponsiveness to single malaria T epitope in highly repetitive vaccine. *J Exp Med* 164:655-60.

Hanna, N., E. Jarosch and S. Leskowitz. 1972. Altered immunogenicity produced by change in mode of linkage of hapten to carrier. *Proc Soc Exp Biol Med* 140:89-92.

Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian and G. Newport. 1987. A major immunogen in Schistosoma mansoni infections is homologous to the heat-shock protein Hsp70. *J Exp Med* 165:1430-5.

Herzenberg, L. A., T. Tokuhisa and L. A. Herzenberg. 1980. Carrier-priming leads to hapten-specific suppression. *Nature* 285:664-7.

Katz, D. H., T. Hamaoka and B. Benacerraf. 1973. Cell interactions between histoincompatible T and B lymphocytes. II. Failure of physiologic cooperative interactions between T and B lymphocytes from allogeneic donor strains in humoral response to hapten-protein conjugates. *J Exp Med* 137:1405-18.

Kaufmann, S. H. E. and B. Schoel. 1994b. Heat shock proteins as antigens in immunity against infection and self. In *The biology of heat shock proteins and molecular chaperones*, R. I. Morimoto, A. Tissieres and C. Georgopoulos, eds. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., p. 495-531.

Kenney, J. S., B. W. Hughes, M. P. Masada and A. C. Allison. 1989. Influence of adjuvants on the quantity, affinity, isotype and epitope specificity of murine antibodies. *J Immunol Methods* 121:157-66.

Kindred, B. and D. C. Shreffler. 1972. H-2 dependence of co-operation between T and B cells in vivo. *J Immunol* 109:940-3.

Klaus, G. G. and A. M. Cross. 1974. The influence of epitope density on the immunological properties of hapten-protein conjugates. I. Characteristics of the immune response to hapten-coupled albumen with varying epitope density. *Cell Immunol* 14:226-41.

Lachmann, P. J. and H. E. Amos. 1970. Soluble factirs in the mediation of the cooperative effect. In *Immunopathology*, P. A. Miescher, eds. Basel, p. 65.

Lachmann, P. J. and K. Sikora. 1978. Coupling PPD to tumour cells enhances their antigenicity in BCG-primed mice. *Nature* 271:463-4.

Lachmann, P. J., L. Strangeways, A. Vyakarnam and G. Evan. 1986. Raising antibodies by coupling peptides to PPD and immunizing BCG-sensitized animals. *Ciba Found Symp* 119:25-57.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-5.

Landsteiner, K. 1936. The specificity of serological reactions. Dover Publications, New York. pp.

Lise, L. D., D. Mazier, M. Jolivet, F. Audibert, L. Chedid and D. Schlesinger. 1987. Enhanced epitopic response to a synthetic human malarial peptide by preimmunization with tetanus toxoid carrier. *Infect Immun* 55:2658-61.

Lowenadler, B. and N. Lycke. 1994. Fusion proteins with heterologous T helper epitopes. Recombinant E. coli heat-stable enterotoxin proteins. *Int Rev Immunol* 11:103-11.

Lowenadler, B., N. Lycke, C. Svanholm, A. M. Svennerholm, K. Krook and M. Gidlund. 1992. T and B cell responses to chimeric proteins containing heterologous T helper epitopes inserted at different positions. *Mol Immunol* 29:1185-90.

Lussow, A. R., M. T. Aguado, G. Del Giudice and P. H. Lambert. 1990a. Towards vaccine optimisation. *Immunol Lett* 25:255-63.

Lussow, A. R., C. Barrios, J. van Embden, R. Van der Zee, A. S. Verdini, A. Pessi, J. A. Louis, P. H. Lambert and G. Del Giudice. 1991. Mycobacterial heat-shock proteins as carrier molecules. *Eur J Immunol* 21:2297-302.

Lussow, A. R., G. Del Giudice, L. Renia, D. Mazier, J. P. Verhave, A. S. Verdini, A. Pessi, J. A. Louis and P. H. Lambert. 1990b. Use of a tuberculin purified protein derivative--Asn-Ala-Asn-Pro conjugate in bacillus Calmette-Guerin primed mice overcomes H-2 restriction of the antibody response and avoids the need for adjuvants. *Proc Natl Acad Sci U S A* 87:2960-4.

Mark, A., B. Bjorksten and M. Granstrom. 1995. Immunoglobulin E responses to diptheria and tetanus toxoids after booster with aluminum-adsorbed and fluid DT-vaccines. *Vaccine* 13:669-673.

McReynolds, L., B. W. O'Malley, A. D. Nisbet, J. E. Fothergill, D. Givol, S. Fields, M. Robertson and G. G. Brownlee. 1978. Sequence of chicken ovalbumin mRNA. *Nature* 273:723-8.

McReynolds, L. A., J. J. Monahan, D. W. Bendure, S. L. Woo, G. V. Paddock, W. Salser, J. Dorson, R. E. Moses and B. W. O'Malley. 1977. The ovalbumin gene. Insertion of ovalbumin gene sequences in chimeric bacterial plasmids. *J Biol Chem* 252:1840-3.

Miller, J. F. and G. F. Mitchell. 1967. The thymus and the precursors of antigen reactive cells. *Nature* 216:659-63.

Mitchison, N. A. 1971a. The carrier effect in the secondary response to hapten-protein conjugates. I. Measurement of the effect with transferred cells and objections to the local environment hypothesis. *Eur J Immunol* 1:10-17.

Mitchison, N. A. 1971b. The carrier effect in the secondary response to hapten-protein conjugates. II. Cellular cooperation. *Eur J Immunol* 1:18-27.

Mosmann, T. R. and R. L. Coffman. 1989. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Ann. Rev. Immunol.* 7:145-73.

Murray, P. J. and R. A. Young. 1992. Stress and immunological recognition in host-pathogen interactions. *J Bacteriol* 174:4193-6.

Nicklas, W. 1992. Aluminum salts. *Res Immunol* 143:489-94.

Ovary, Z. and B. Benacerraf. 1963. Immunological specificity of the secondary response with dinitrophenylated proteins. *Proc Soc Exp Biol Med* 114:72-76.

Perraut, R., A. R. Lussow, S. Gavoille, O. Garraud, H. Matile, C. Tougne, J. Van Embden, R. Van der Zee, P. H. Lambert, J. Gysin and G. Del Giudice. 1993. Successful primate immunization with peptides conjugated to purified protein derivative or mycobacterial heat shock proteins in the absence of adjuvants. *Clin. Exp. Immunol.* 93:382-386.

Raff, M. C. 1970. Role of thymus-derived lymphocytes in the secondary humoral immune response in mice. *Nature* 226:1257-8.

Rajewsky, K., V. Schirrmacher, S. Nase and N. K. Jerne. 1969. The requirement of more than one antigenic determinant for immunogenicity. *J Exp Med* 129:1131-43.

Robbins, J. B. and R. Schneerson. 1990. Polysaccharide-protein conjugates: a new generation of vaccines. *J Infect Dis* 161:821-32.

Rothstein, N. M., G. Higashi, J. Yates and T. V. Rajan. 1989. Onchocerca volvulus heat shock protein 70 is a major immunogen in amicrofilaremic individuals from a filariasis-endemic area. *Mol Biochem Parasitol* 33:229-35.

Selkirk, M. E., D. A. Denham, F. Partono and R. M. Maizels. 1989. Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. *J Immunol* 143:299-308.

Smith, D. H., D. V. Madore, R. J. Eby, P. W. Anderson, R. A. Insel and C. L. Johnson. 1989. Haemophilus b oligosaccharide-CRM197 and other Haemophilus b conjugate vaccines: a status report. *Adv Exp Med Biol* 251:65-82.

Snippe, H., W. G. Graven and P. J. Willems. 1975. Antibody formation in the mouse induced by hapten-carrier complexes. *Immunology* 28:885-95.

Studier, F. W., A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185:60-89.

Suzue, K. and R. A. Young. 1996. Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. *J Immunol* 156:873-9.

Tabor, S. and C. C. Richardson. 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. *Proc Natl Acad Sci U S A* 82:1074-8.

Vanbuskirk, A., B. L. Crump, E. Margoliash and S. K. Pierce. 1989. A peptide binding protein having a role in antigen presentation is a member of the HSP70 heat shock family. *J Exp Med* 170:1799-809.

Young, D., R. Lathigra, R. Hendrix, D. Sweetser and R. A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A* 85:4267-70.

Young, D. B., L. Kent and R. A. Young. 1987. Screening of a recombinant mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens. *Infect Immun* 55:1421-5.

Section III

Generation of CTL Responses.

Section III: Generation of CTL Responses

The ability to induce antigen-specific CTL responses is of broad interest due to the essential role of CTLs in protective cellular immune responses, including the elimination of virally infected cells (Kast et al., 1986; Lin and Askonas, 1981; Lukacher et al., 1984). CTL induction is dependent upon the presentation of processed antigen to naive T cells by professional antigen presenting cells (APCs) that express high levels of class I major histocompatibility complex (MHC) molecules (Townsend and Bodmer, 1989; Townsend and Allison, 1993) . In chapter 5, I will discuss antigen presentation pathways of exogenous and endogenous antigens. In chapter 6, I will then describe my work which demonstrates that an hsp70 fusion protein administered in the absence of adjuvant can elicit CTLs and induce protective tumor immunity in mice. The work described in chapter 6 has been published as: Suzue, K., Zhou, X., Eisen, H.N. and Young, R.A., Heat shock fusion proteins as vehicles for antigen delivery into the major histocompatibility complex class I presentation pathway. Proc Natl Acad Sci U SA 94: 13146-13151 (1997).

Section III

Chapter 5. Antigen Presentation

MHC class I and class II restricted antigen presentation are typically described as two distinct pathways which differ in the source of antigen (cytosolic vs. extracellular) and differ in the cellular compartments which are involved (Braciale et al., 1987; Brodsky and Guagliardi, 1991; Morrison et al., 1986). In the conventional MHC class I antigen presentation pathway, cytosolic antigens are proteolyzed in the cytoplasm by the multicatalytic protease, the proteasome (Driscoll and Finley, 1992; Goldberg and Rock, 1992). The cytosolic peptides are transported into the endoplasmic reticulum (ER) by the peptide transporter associated with antigen presentation (TAP) (Androlewicz et al., 1993; Neefjes et al., 1993; Shepherd et al., 1993). In the ER lumen, the peptides bind to MHC class I molecules and form a complex which migrates to the cell surface for recognition and activation of $CD8^+$ CTL. In contrast to cytosolic antigens, extracellular protein antigens are normally endocytosed into intracellular vesicles and subsequently proteolyzed by cathepsins (Chapman, 1998). The peptides generated can bind to MHC class II molecules trafficking in the endocytic route (Xu and Pierce, 1995) and the resulting complex is then presented at the cell surface to $CD4^+$ helper T cells (Wolf and Ploegh, 1995).

Extensive experimental data supports this basic model whereby cytosolic antigens or any protein in the topological equivalent of extracellular space are usually shuttled into the MHC class I pathway while exogenous antigens are processed in the MHC class II pathway. For example, experiments by Braciale et al. demonstrated that the addition of viable virus

preparations to cells allowed the synthesis of viral antigen within the cytosol of the antigen presenting cell and resulted in viral peptide presentation on the cell surface by MHC class I molecules (Braciale, et al., 1987). In contrast, nonviable viral antigens were endocytosed and processed by a chloroquine-inhibited mechanism which resulted in the processing of viral antigens for presentation by MHC class II molecules. Other investigators have demonstrated that exogenously added protein can be processed for class I restricted presentation when artificially delivered into the cytosolic compartment, for example as a consequence of the osmotic lysis of pinosomes (Moore et al., 1988), membrane fusion (Yewdell et al., 1988) or electroporation (Harding, 1992).

Alternative MHC class I presentation pathways

Despite the general view that the MHC class I pathway exclusively monitors the endogenously synthesized proteins of a cell, it now appears that exogenous antigens can also gain access to the MHC class I antigen presentation pathway (Jondal et al., 1996; Rock, 1996). Early studies by Bevan described an *in vivo* "cross-priming" phenomenon where transfer of donor H-2^b cells into F1 H-2 ^{bxd} mice generated H-2^d restricted alloreactive CTL. This response apparently resulted from the presentation of H-2^b donor cell-derived alloantigen peptides by H-2^d MHC molecules expressed by antigen presenting cells native to the recipient animals (Bevan, 1976). Subsequent studies continue to demonstrate that cell-associated antigens can be taken up, processed, and then presented to CTL precursors by host cells (Arnold et al., 1995; Huang et al., 1996).

Furthermore, proteins such as ovalbumin, hepatitis B surface antigen and HIV gp120 can be administered as exogenous antigen preparations in

order to prime MHC class I restricted T cell responses *in vivo* (Bohm et al., 1995; Raychaudhuri et al., 1992; Schirmbeck et al., 1995; Wijburg et al., 1998). Indeed, although many investigators have been unable to prime anti-ovalbumin CTL *in vivo* by administering soluble ovalbumin protein in saline, CTLs against ovalbumin have been elicited by administering denatured ovalbumin (Martinez-Kinader et al., 1995; Schirmbeck et al., 1994a) ovalbumin coupled to beads (Harding and Song, 1994; Kovacsovics-Bankowski et al., 1993), cell-associated ovalbumin (Carbone and Bevan, 1990) or recombinant bacteria expressing ovalbumin (Pfeifer et al., 1993). In addition, formulations of ovalbumin with adjuvants such as complete Freund's adjuvant, saponin, squalene and Tween 80 elicited antigen-specific CTL responses (Ke et al., 1995; Newman et al., 1992; Raychaudhuri, et al., 1992).

The predominant mechanism by which peptides derived from extracellularly added antigens combine with MHC class I molecules is currently unresolved. In some cases, exogenous antigen fragments may escape endocytic vesicles and enter the cytoplasm, merging with the conventional MHC class I pathway. For example, macrophage from wild type mice processed ovalbumin coupled to latex particles in the context of MHC class I molecules (Kovacsovics-Bankowski and Rock, 1995). However, the use of macrophage from TAP1 mutant mice or the addition of proteosomal inhibitors to wild type macrophage blocked the presentation of the exogenous antigen with MHC class I. The failure of chloroquine to inhibit presentation indicates that the antigen is processed in a nonlysosomal compartment and further suggests the trafficking of the exogenous antigen into the conventional MHC class I presentation pathway. The transfer of exogenous material from endocytic compartments into the cytosol has been visualized *in*

vitro by incubating bone marrow macrophage with horseradish peroxidase or fluorescein isothiocyanate-dextran (Norbury et al., 1995).

Other studies have illustrated independence of exogenous antigens from conventional cytosolic processing mechanisms and ER function. The phagocytic processing of *E. coli* or *Salmonella typhimurium* expressing ovalbumin resulted in MHC class I presentation of ovalbumin in a brefeldin A resistant pathway (Pfeifer, et al., 1993). Ovalbumin conjugated to beads has also been reported to be processed and presented in the context of MHC class I by macrophage in a brefeldin A resistant pathway (Harding and Song, 1994). In studies utilizing recombinant glyco- and nucleoprotein from lymphocytic choriomeningitis virus and nucleoprotein of vesicular stomatitis virus, viral peptides were presented in association with class I MHC molecules. The presentation was as efficient in TAP deficient macrophage as by wild type macrophage (Bachmann et al., 1995).

It should be emphasized that the quality of the antigen preparation may have a large impact on the measurement of MHC class I responses *in vivo* and *in vitro*. For example, Sousa and Germain (1995) screened nine different batches of commercially purchased ovalbumin and found that seven of the batches resulted in the formation of ovalbumin peptide SIINFEKL bound to MHC class I on the surface of paraformaldehyde-fixed antigen presenting cells, in the absence of intracellular antigen presentation. Sousa and Germain thus rigorously screened ovalbumin preparations and only utilized two of the nine batches of ovalbumin for their in vitro antigen presentation assays. Protein preparations containing numerous degradation products may significantly affect the result of MHC class I presentation assays.

It should also be noted that antigen presenting cells process particulate antigens much more efficiently than soluble antigens (Harding and Song,

1994; Pfeifer, et al., 1993; Rock, 1996). Ovalbumin coupled to latex particles was processed by macrophage *in vitro* and presented by MHC class I molecules 100 to 1000-fold more efficiently than soluble ovalbumin (Harding and Song, 1994). Numerous published results demonstrate that injection of 1 μg to 1 mg of native soluble ovalbumin protein in saline solution into mice fails to elicit an anti-ovalbumin CTL response (Ke, et al., 1995; Martinez-Kinader, et al., 1995; Moore, et al., 1988; Raychaudhuri, et al., 1992; Schirmbeck, et al., 1994a). Studies which do demonstrate the class I presentation of exogenous soluble antigen *in vitro* have used very high concentrations of antigen, from 4 to 10 mg/ml (Norbury, et al., 1995; Reis e Sousa and Germain, 1995; Rock et al., 1993).

Investigators have utilized different cell types as well as a broad range of antigen formulations in *in vitro* antigen presentation assays. Both macrophages and dendritic cells have prominent phagocytic and macropinocytic activity and are capable of mediating class I presentation of exogenous antigens (Albert et al., 1998; Norbury et al., 1997; Rock, et al., 1993). The mechanism by which these specialized antigen presenting cells process exogenous antigens is currently an area of intense investigation.

In summary, exogenous antigens have been found to prime MHC class I restricted CTL responses under certain circumstances and various antigen formulations appear to be handled differently by antigen presenting cells. In contrast to particulate antigens, the efficient MHC class I presentation of soluble protein antigens is infrequently observed. Thus, as described in the next chapter, I examined whether the hsp70 could be utilized to enhance the delivery of an antigen into the MHC class I presentation pathway.

Section III

Chapter 6.

Heat shock proteins as vehicles for antigen delivery into the MHC class I presentation pathway

Summary

Mice immunized with heat shock proteins (hsp) isolated from mouse tumor cells (donor cells) produce CD8 cytotoxic T lymphocytes (CTL) that recognize donor cell peptides in association with the MHC class I proteins of the responding mouse. The CTL are induced apparently because peptides noncovalently associated with the isolated hsp molecules can enter the MHC class I antigen processing pathway of professional antigen presenting cells. Using a recombinant heat shock fusion protein with a large fragment of ovalbumin covalently linked to mycobacterial hsp70, we show here that when the soluble fusion protein was injected without adjuvant into H-2^b mice, CTL were produced that recognized an ovalbumin-derived peptide, SIINFEKL, in association with K^b. The peptide is known to arise from natural processing of ovalbumin in H-2^b mouse cells, and both CTL from the ova-hsp70-immunized mice and a highly effective CTL clone (4G3) raised against ovalbumin-expressing EL4 tumor cells (EG7-OVA), were equally effective in terms of the concentration of SIINFEKL required for half-maximal lysis in a CTL assay. The mice were also protected against lethal challenge with ovalbumin-expressing melanoma tumor cells. Because large protein fragments or whole proteins serving as fusion partners can be cleaved into short peptides in the MHC class I processing pathway, hsp fusion

proteins of the type described here are promising candidates for vaccines aimed at eliciting CD8 CTL in populations of MHC-disparate individuals.

Introduction

The cytotoxic T lymphocytes (CTL) that play an important role in protective cellular immunity, including the destruction of virus-infected cells, are predominantly CD8 T cells (Byrne and Oldstone, 1984; Nagler-Anderson et al., 1988). Antigen-specific activation of these cells depends upon their recognition of peptide-MHC complexes, which normally arise within antigen presenting cells by proteolytic cleavage of cytosolic proteins (Townsend and Bodmer, 1989). Translocated into the ER, the resulting peptides bind to nascent class I MHC molecules for transport to the cell surface (Heemels and Ploegh, 1995). Since intact proteins in the extracellular medium do not ordinarily penetrate into a cell's cytosol, soluble proteins typically fail to stimulate mice to produce CTL (Braciale, et al., 1987), although there are exceptions (Jondal, et al., 1996).

In comparison with other proteins, the soluble heat shock protein termed gp96 is an unusually effective stimulator of CD8 CTL (Udono and Srivastava, 1994). Mice injected with gp96 isolated from tumor cells (donor cells) produce CTL that are specific for donor cell peptides in association with the responder mouse's class I MHC proteins (Arnold, et al., 1995; Udono and Srivastava, 1994). Since donor peptides are bound noncovalently by the isolated hsp protein, the results suggest that the hsp molecules are capable of delivering noncovalently associated peptides to MHC-I proteins of other (recipient) cells, including antigen presenting cells.

The noncovalently bound peptide-gp96 complexes which are purified from a tumor cell appear to represent a broad array of proteins expressed by the cell (Arnold et al., 1997; Li and Srivastava, 1993). In contrast, recombinant hsp fusion proteins in which specific proteins of interest are covalently linked to the hsp would provide a well-characterized polypeptide which would lack

extraneous peptides. In addition, a large protein fragment covalently linked to the hsp would be an especially rich source of many different naturally processed peptides. Peptide mixtures of this kind, derived from specific antigens of interest, would be particularly suitable for forming intracellular peptide-MHC complexes with the highly diverse MHC proteins found in different individuals of genetically outbred populations.

We have accordingly taken advantage of a recombinant hsp70 protein expression vector that permits diverse proteins and peptides to be fused to the amino terminus of mycobacterial hsp70. We have previously shown that *M. tuberculosis* hsp70 can be used as an adjuvant-free carrier to stimulate the humoral and cellular response to a full-length protein that is covalently linked to the hsp (Suzue and Young, 1996) . The special properties of hsp70 prompted us to investigate whether soluble hsp70 fusion proteins could be utilized to elicit MHC class-I restricted $CD8^+$ CTL.

We show here that a soluble hsp70 fusion protein having a large fragment of chicken ovalbumin as fusion partner could, in the absence of adjuvants, stimulate H-2^b mice to produce ovalbumin-specific CD8 CTL. The CTL recognized an immunodominant ovalbumin octapeptide, SIINFEKL, known to be a naturally processed peptide derived from ovalbumin expressed in mouse cells (Rotzschke et al., 1991), in the context of K^b. CTL from the immunized mice were as active cytolytically as a highly effective CTL clone (4G3) that had been raised against ovalbumin-expressing tumor cells, as both caused half-maximal lysis of K^{b+} target cells with the SIINFEKL peptide at about the same concentration
(10^{-13} M). Our results thus indicate that the ovalbumin-hsp70 fusion protein, injected as a soluble protein into mice, can enter the MHC class I processing pathway in antigen presenting cells and stimulate the production of CD8 CTL.

Results

Purified recombinant proteins

A recombinant system developed to permit production of *M. tuberculosis* hsp70 fusion proteins in *E. coli* (Suzue and Young, 1996) was utilized to attach amino acids 161 to 276 of ovalbumin to the N-terminus of *M. tuberculosis* hsp70. A comparable recombinant ovalbumin protein (amino acids 161 to 276) was also produced. The selected portion of ovalbumin contains the immunodominant epitope SIINFEKL recognized by CTL in association with K^b (Carbone and Bevan, 1989; Rotzschke, et al., 1991). The ovalbumin-hsp70 fusion protein and the ovalbumin (aa 161-276) protein were expressed at high levels in *E. coli* (Fig. 1A). These proteins were purified as inclusion bodies, refolded *in vitro*, and further purified by column chromatography. The purity of the recombinant proteins was assessed by SDS-PAGE (Fig. 1A). Examination of commercial preparations of crystallized and high grade ovalbumin by SDS-PAGE and silver staining revealed that they are highly contaminated with low molecular weight polypeptides. For this reason, only the highly purified recombinant ovalbumin (aa 161-276) protein, referred to below simply as ovalbumin, was used in these studies.

Figure 1

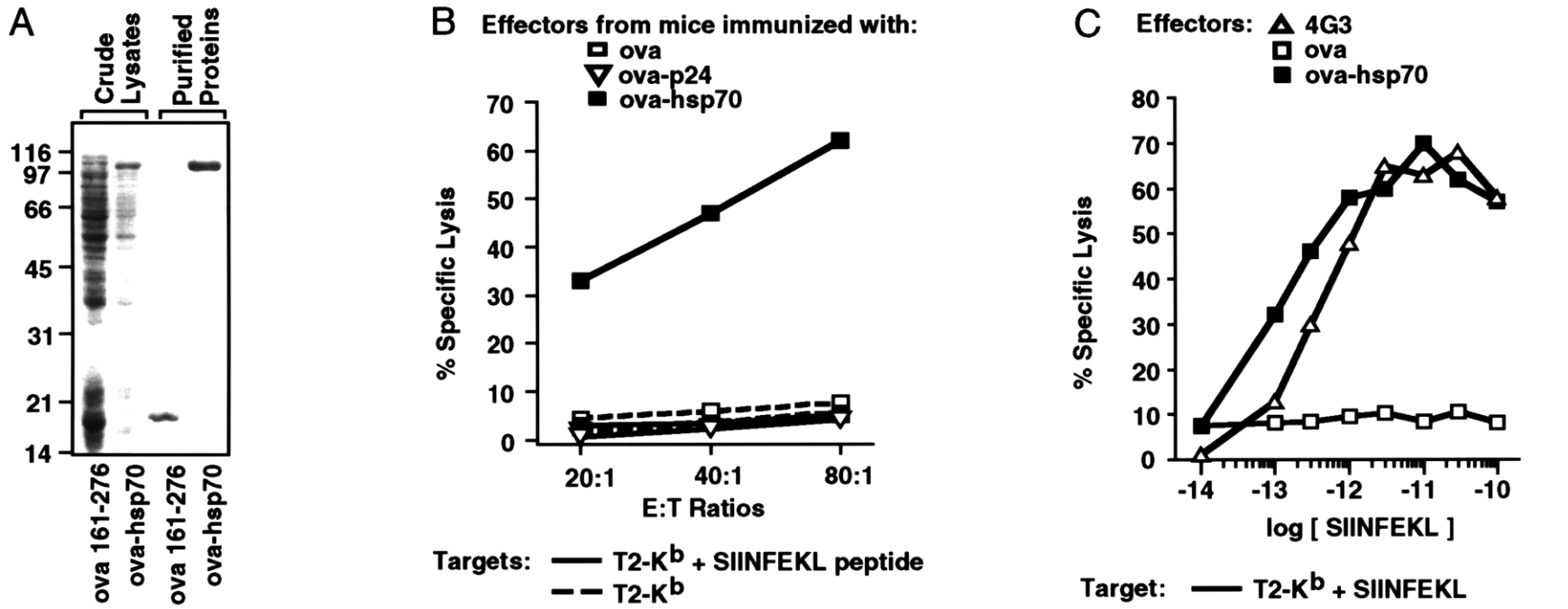

A
Crude Lysates
Purified Proteins
116
97
66
45
31
21
14
ova 161-276
ova-hsp70
ova 161-276
ova-hsp70
B
Effectors from mice immunized with:
ova
ova-p24
ova-hsp70
% Specific Lysis
70
60
50
40
30
20
10
0
20:1
40:1
80:1
E:T Ratios
Targets: T2-K^b + SIINFEKL peptide
T2-K^b
C
Effectors: 4G3
ova
ova-hsp70
% Specific Lysis
80
70
60
50
40
30
20
10
0
-14
-13
-12
-11
-10
log [SIINFEKL]
Target: T2-K^b + SIINFEKL

Fig. 1. A) Production of recombinant proteins. *E. coli* cell lysates and purified proteins were examined by SDS-PAGE and proteins were visualized by Coomassie staining. The gel contains crude extracts from IPTG-induced *E. coli* containing pKS28 (ova 161-276) and from IPTG-induced *E. coli* containing pKS76 (ova-hsp70), and the purified proteins ova 161-276 and ova-hsp70. Molecular weight markers ($X10^{-3}$) are at left. B) Generation of ovalbumin-specific CTL by immunization with ova-hsp70 fusion protein in saline. Mice were injected i.p. with either 120 pmoles of recombinant ova, ova-p24 or ova-hsp70 protein without adjuvant. The injections were repeated s.c. 2 weeks later. Mice were sacrificed 10 days after the boost and for each mouse group, 5-10 spleens were pooled and splenocytes from immunized mice were incubated for 6 days in the presence of irradiated E.G7-OVA cells without added interleukins. The splenocyte cultures derived from mice immunized with ova □, ova-p24 ▽ and ova-hsp70 ■ were then used as effector cells in a standard cytotoxicity assay. The following ^{51}Cr-labeled target cells were used: T2-K^b cells — — and T2-K^b pulsed with SIINFEKL peptide ——— at 300 μg/ml. C) SIINFEKL peptide titration. T2-K^b cells were incubated with the indicated molar concentrations of SIINFEKL peptide for 45 min. for use as target cells in a CTL assay. The effector cells ova □ and ova-hsp70 ■ as described above, were used at an E:T ratio of 80:1. The 4G3 CTL clone Δ was used at an E:T of 5:1.

Immunization of mice with hsp70 fusion protein in PBS elicits T cell responses against the attached antigen

We investigated whether mice injected with soluble protein without adjuvant could be primed to produce anti-ovalbumin T cells (Fig. 1B). C57BL/6 mice were inoculated i.p. with 120 pmoles of ovalbumin or with 120 pmoles of ovalbumin-hsp70 fusion protein in PBS. A second equivalent dose was given s.c. at two weeks. A third group of mice was injected with 120 pmoles of ovalbumin-p24 gag fusion protein, purified as described in (Suzue and Young, 1996), in order to examine the immune responses elicited by administering ovalbumin covalently linked to a protein other than hsp70, in the absence of adjuvant. Splenocytes of immunized mice were removed ten days after the s.c. immunization and cultured *in vitro* for 6 days with irradiated E.G7-OVA cells (syngeneic EL4 cells transfected with ovalbumin) (Moore, et al., 1988). The cultured cells were then used as effector cells in CTL assays. Cells from mice injected with ovalbumin protein or with ovalbumin-p24 fusion protein were unable to lyse T2-K^b target cells or T2-K^b cells pulsed with SIINFEKL peptide. In contrast, effector cells from mice primed with ovalbumin-hsp70 fusion protein were able to lyse T2-K^b cells pulsed with SIINFEKL peptide (Fig. 1B).

Results obtained with other target cells also show that the anti-ovalbumin CTL recognized SIINFEKL in association with K^b. Splenocytes from ovalbumin-hsp70 immunized mice were able to lyse both E.G7-OVA target cells and EL4 cells pulsed with SIINFEKL peptide but were unable to lyse EL4 cells in the absence of peptide or EL4 cells pulsed with another K^b-binding peptide (RGYVYQGL, from vesicular stomatitis virus, (Van Bleek and Nathenson, 1990), data not shown).

To assess the effectiveness of the CTL from ova-hsp70-immunized mice, they were tested after 6 days in culture in cytolytic assays using T2-K^b as target cells and SIINFEKL at various concentrations. For purposes of comparison, the assay included a well-characterized CTL clone (4G3) that recognizes the SIINFEKL-K^b complex. As shown in Fig. 1C, half-maximal lysis was obtained with both the CTL line and the 4G3 clone at about the same peptide concentration, approximately 5×10^{-13} M. Thus CTL from the ova-hsp70-immunized mice and the clone against the ovalbumin-expressing tumor (E.G7-OVA) were equally effective in terms of the SIINFEKL concentration required for half-maximal lysis. It may be noted that in Fig. 1C the ratio of 4G3 cells to target cells (E:T ratio) was 5:1, whereas for the CTL line this ratio was 80:1. While the E:T ratio has a large impact on the maximal lysis of target cells at 4 hr, changing this ratio over an 80-fold range (1:1 to 80:1) has a negligible effect on the peptide concentration required for half-maximal lysis (unpublished data).

We next verified that the cytolytic activity of the CTL line from ova-hsp70-immunized mice was due to $CD8^+$ T cells (Fig. 2). For this purpose we used a MACS column to separate the CTL line into T cell subsets (see Materials and Methods). CTL activity was unaffected by removing $CD4^+$ cells, but it was completely abrogated by removing $CD8^+$ cells. Retrieval of the $CD8^+$ cells from the MACS column led to recovery of cytolytic activity. The results were the same when target cells were EL4 cells incubated with SIINFEKL or ovalbumin-expressing EL4 cells (E.G7-OVA). Thus, administration of ovalbumin-hsp70 fusion protein, but not ovalbumin alone, elicits $CD8^+$ CTL specific for SIINFEKL-K^b.

Figure 2

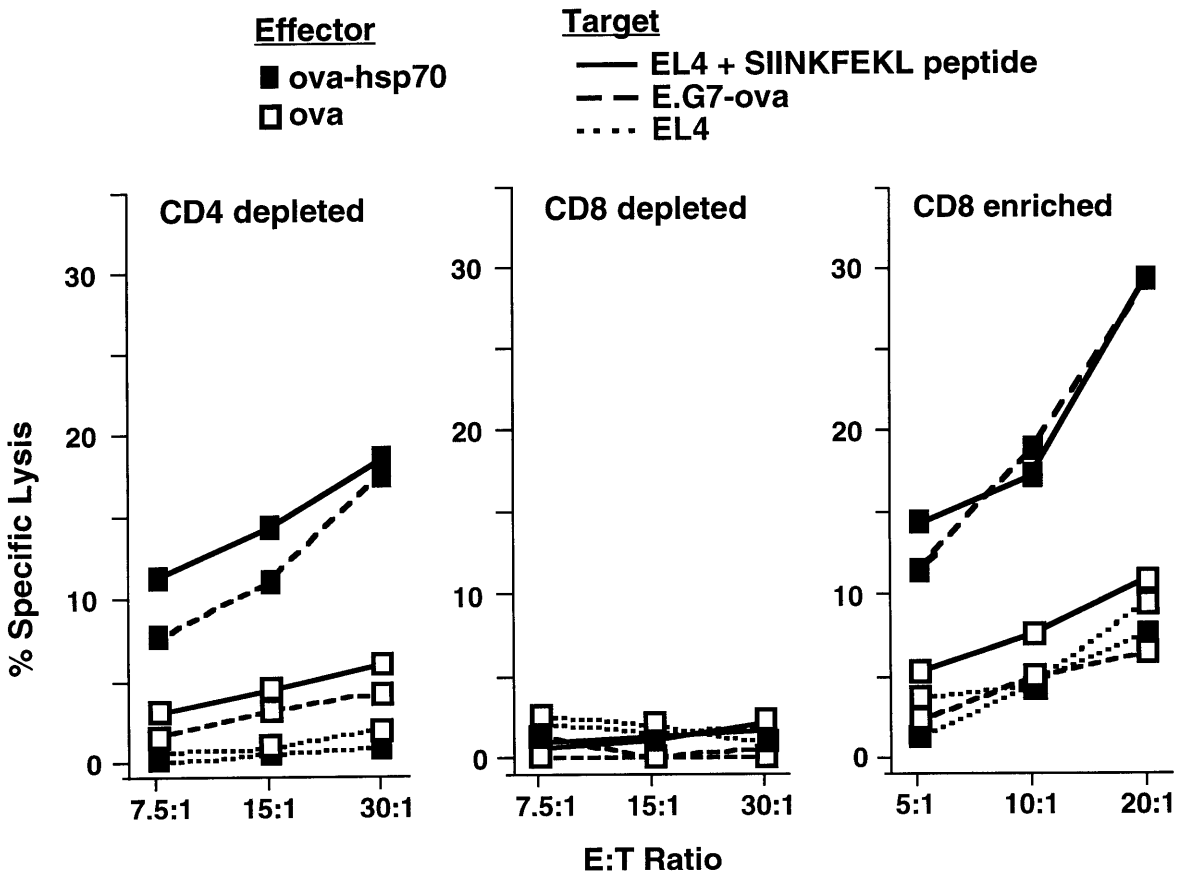

Effector
ova-hsp70
ova
Target
EL4 + SIINKFEKL peptide
E.G7-ova
EL4
CD4 depleted
CD8 depleted
CD8 enriched
% Specific Lysis
0
10
20
30
7.5:1
15:1
30:1
5:1
10:1
20:1
E:T Ratio

Fig. 2. Immunization with ova-hsp70 elicits ovalbumin reactive $CD8^+$ T cells. C57BL/6 mice were injected i.p. with 120 pmoles of ova or ova-hsp70 without adjuvant and boosted s.c. with the same amounts of these proteins 2 weeks later. Mice were sacrificed 10 days after the boost and for each mouse group, 5-10 spleens were pooled and splenocytes were incubated for 6 days in the presence of irradiated E.G7-OVA cells. Prior to performing the cytotoxicity assay, the effector cells were negatively or positively selected for $CD4^+$ cells or $CD8^+$ cells using paramagnetic antibodies (see Materials and Methods). Splenocyte cultures were either depleted of $CD4^+$ cells ($CD4^-CD8^+$), depleted of $CD8^+$ cells ($CD4^+$ $CD8^-$) or were enriched for $CD8^+$ cells ($CD8^+$).

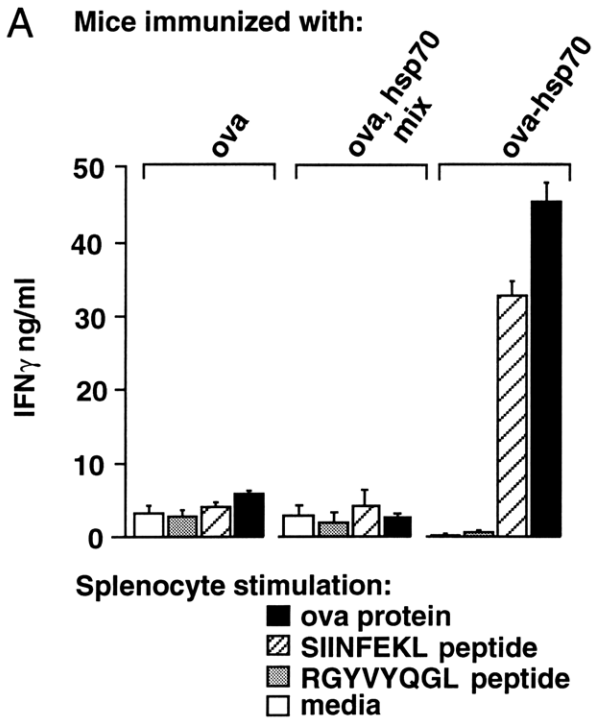
The lower level of cytolytic activity in Fig. 2 relative to Fig. 1 (B and C) reflects the different target cells used. T2-K^b cells (Fig. 1) and EL4 cells (Fig. 2) have approximately the same high level of cell surface K^b (roughly 100,000 molecules per cell, unpublished observations), but the peptide transporter (TAP) is defective in T2-K^b (Andersen and Heron, 1993), and not in EL4. Hence, at a given free concentration of SIINFEKL the target cell epitope density (number of SIINFEKL-K^b complexes per cell) is much greater on T2-Kb than EL4 cells.

Hsp70 must be covalently coupled to ovalbumin to engender anti-ovalbumin T cell responses

We examined whether the covalent fusion of hsp70 to ovalbumin was necessary to elicit cellular responses to ovalbumin or whether the same results could be obtained if the two proteins were simply mixed but not covalently attached (Fig. 3). Mice were injected with 120 pmoles of ovalbumin-hsp70 fusion protein, with 120 pmoles of ovalbumin, or with 120 pmoles of hsp70 mixed with 120 pmoles of ovalbumin. The level of IFN-γ secreted by the splenocytes in response to restimulation with ovalbumin *in vitro* was measured by ELISA. Splenocytes from mice immunized with ovalbumin alone or with a mixture of ovalbumin and hsp70 proteins produced less than 6 ng/ml IFN-γ in response to stimulation with SIINFEKL peptide or ovalbumin (Fig. 3A). In contrast, splenocytes from mice injected with the ovalbumin-hsp70 fusion protein secreted substantially higher levels of IFN-γ when restimulated *in vitro* with SIINFEKL peptide or ovalbumin. The release of IFN-γ was ovalbumin-specific, since splenocytes cultured in media alone or with control RGYVYQGL peptide secreted low levels of IFN-γ.

Similar results were obtained by cytolytic assays (Fig. 3B). Ovalbumin-specific CTL were produced by mice injected with the ovalbumin-hsp70 fusion protein but not by those injected with a mixture of ovalbumin with hsp70.

Figure 3


A
Mice immunized with:
ova
ova, hsp70 mix
ova-hsp70
IFNγ ng/ml
50
40
30
20
10
0
Splenocyte stimulation:
ova protein
SIINFEKL peptide
RGYVYQGL peptide
media

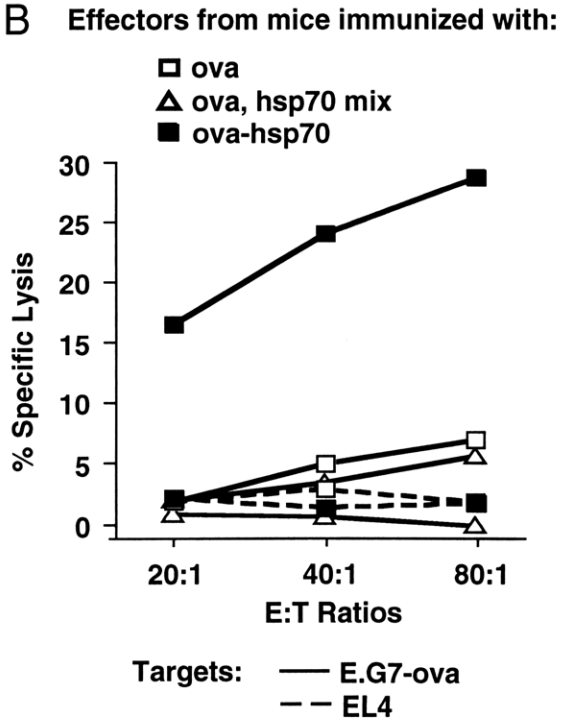

B
Effectors from mice immunized with:
ova
ova, hsp70 mix
ova-hsp70
% Specific Lysis
30
25
20
15
10
5
0
20:1
40:1
80:1
E:T Ratios
Targets:
E.G7-ova
EL4

Fig. 3. Examination of ovalbumin-specific T cell responses in mice injected with a mixture of ova and hsp70. Mice were injected twice as described in Fig. 1 with 120 pmoles of recombinant ova, 120 pmoles of ova-hsp70 fusion protein or with 120 pmoles each of ova and hsp70. Ten days after the boost 5-10 spleens from each mouse group were pooled and processed. A) IFN-γ secretion by splenocytes stimulated 72 h *in vitro* with 5 μg/ml recombinant ova protein ■, SIINFEKL peptide ▨, RGYVYQGL peptide ▩, or tissue culture media alone □. All samples were examined in triplicate. B) Generation of ova-specific CTL by immunization with ova-hsp70 fusion protein in saline. Splenocyte cultures from mice immunized with recombinant ova ◘, ova-hsp70 fusion protein ■ or with a mixture of ova and hsp70 proteins Δ, were used as effector cells in a standard cytotoxicity assay. The following ^{51}Cr-labeled target cells were used: E.G7-OVA ——— and EL4 cells alone — —.

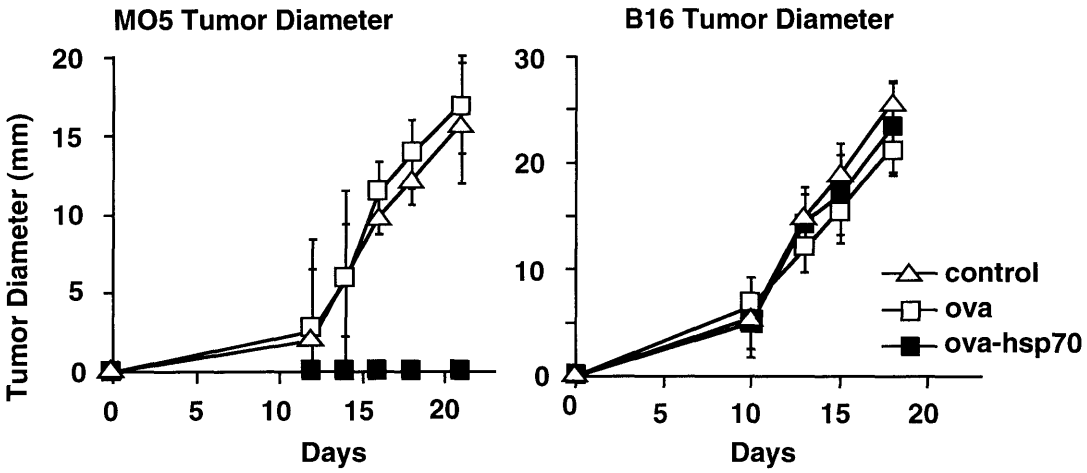
Immunization of mice with ovalbumin-hsp70 protein without adjuvant engenders protective immunity to MO5 tumor challenge

The MO5 cell line, which is a B16 melanoma cell line transfected with ovalbumin expressing DNA, presents the immunodominant SIINFEKL peptide in association with K^b on the cell surface (Falo, et al., 1995). Using this tumor we could determine whether the immune response induced by ovalbumin-hsp70 fusion protein is sufficient to engender protective tumor immunity. Mice were injected i.p. with 120 pmoles of ovalbumin or ovalbumin-hsp70 without adjuvant and boosted s.c. 2 weeks later. Ten days later the mice were injected s.c. on the right flank with 1×10^5 MO5 tumor cells or with 1×10^5 B16 tumor cells. As an additional control, naive mice were also inoculated with the tumor cells.

All mice challenged with tumor cells were monitored for tumor growth and growth was recorded as the average tumor diameter in millimeters (Fig. 4A). Twenty-one days following the MO5 tumor challenge, the average tumor diameter in the control and the ovalbumin immunized mice was greater than 15 mm. Because the control and ovalbumin immunized mice began dying 21 days after the tumor challenge, tumor growth was not recorded beyond 21 days. In contrast to the control and the ovalbumin-immunized mice, no tumors were detected in the ovalbumin-hsp70 immunized mice 21 days after the tumor challenge. All groups of mice (control, ovalbumin-immunized or ovalbumin-hsp70 immunized) which were challenged with the B16 tumor cells developed tumors (Fig. 4A) and became moribund by 21 days after the tumor challenge.

Figure 4

A


MO5 Tumor Diameter
B16 Tumor Diameter
Tumor Diameter (mm)
20
15
10
5
0
30
20
10
0
0
5
10
15
20
Days
control
ova
ova-hsp70

B

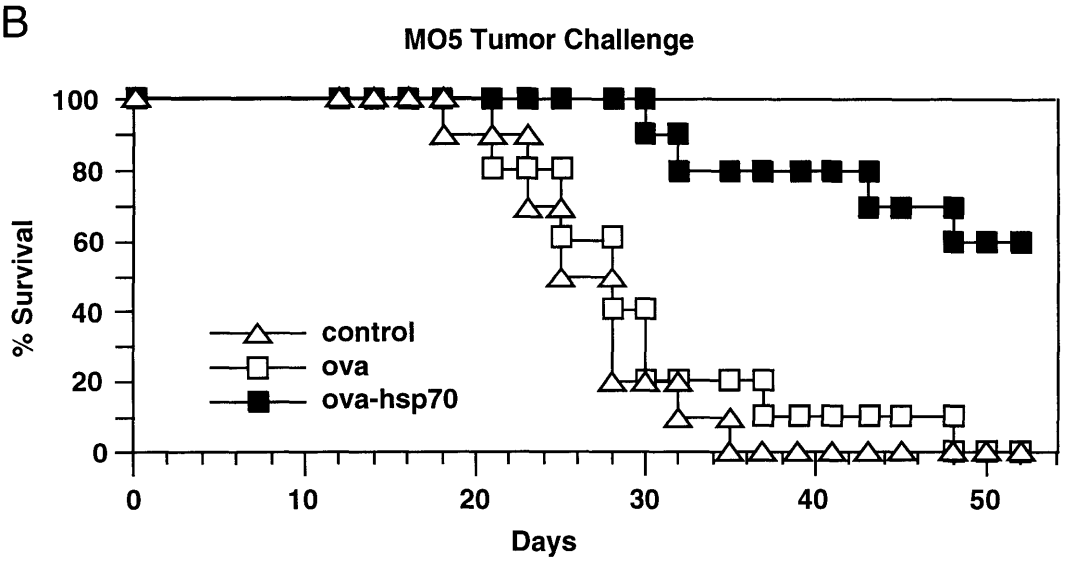

MO5 Tumor Challenge
% Survival
100
80
60
40
20
0
0
10
20
30
40
50
Days
control
ova
ova-hsp70

Fig. 4. Immunization of mice with ova-hsp70 protein without adjuvant engenders protective immunity to MO5 tumor challenge. Mice were injected i.p. with 120 pmoles of ova or ova-hsp70 without adjuvant and boosted s.c. with the same amounts of these proteins 2 weeks later. 10 days after the last immunization the mice were injected s.c. on the right flank with 1 X 10^5 MO5 tumor cells. Each group contained at least 5 mice. A) Following the MO5 and B16 tumor challenges, tumor growth was monitored in control mice △ and in ova □ and ova-hsp70 ■ immunized mice. Growth was recorded as the average tumor diameter in millimeters. B) The survival of mice was recorded as the percentage of mice surviving following the tumor challenge. Mice which appeared moribund were killed and scored as 'not surviving'.

The survival of mice was recorded as the percentage of mice surviving following the tumor challenge (Fig. 4B). Mice which appeared moribund were sacrificed. Forty days after the MO5 tumor challenge, none of the control mice and only 10% of the ovalbumin-immunized mice had survived. In contrast, 80% of the ovalbumin-hsp70 immunized mice had survived. These experiments demonstrate that immunization of mice with the ovalbumin-hsp70 fusion protein, but not with the ovalbumin protein alone, induces ovalbumin specific protective tumor immunity.

Discussion

The principal finding in this study is that injection of an hsp70-ovalbumin fusion protein into H-2^b mice stimulated the production of CD8 CTL that recognize the immunodominant ovalbumin octapeptide, SIINFEKL, in association with Kb. The immunized mice were protected against an otherwise lethal challenge with an ovalbumin-expressing melanoma tumor, and their CTL were as effective (see Fig. 1C) in recognizing the SIINFEKL-K^b complex as a CTL clone (4G3) that was raised against cells (EG7-OVA) in which ovalbumin is expressed and processed naturally for class I-MHC presentation. These findings clearly imply that the covalently linked fusion partner of the injected hsp fusion protein was processed in the same way as ordinary cytosolic proteins for presentation with MHC class I proteins in antigen presenting cells.

We previously reported that mice injected with an HIV-1 gag protein (p24) linked to hsp70 produced p24-specific T cells. Although the peptide-MHC complexes recognized by the T cells were not identified, the splenocytes from the fusion-protein immunized mice exhibited p24 antigen-dependent production of IFN-γ, which implies the presence of Th1 helper T cells and CTL. The previous findings, taken in conjunction with the present results, suggest that hsp70 fusion proteins may prove to be generally useful as immunogens for stimulating CD8 CTL that are specific for peptides produced by natural proteolytic processing of the fusion partners within antigen presenting cells.

The mechanisms by which hsp70 enables covalently linked polypeptide fusion partners to gain entry into the MHC class I processing pathway and elicit CD8 CTL could be based on: i) hsp70's ability to assist protein folding (Flynn et al., 1991; Zhu et al., 1996), and to facilitate the

translocation of proteins into subcellular compartments (Brodsky, 1996; Cyr and Neupert, 1996), ii) hsp70's ability to facilitate the breakdown of intracellular proteins (Sherman and Goldberg, 1996) and iii) the high frequency of T cells directed against mycobacterial hsp70.

Hsp70 is an integral component of the protein folding machinery (Gething and Sambrook, 1992; Hartl, 1996; Hartl et al., 1994) and functions through its ability to bind short linear peptide segments of folding intermediates. Detailed studies of the peptide-binding activity of hsp70 have shown that it has a clear preference for peptides with aliphatic hydrophobic side chains (Flynn, et al., 1991; Rudiger et al., 1997). Thus hsp70 appears to transiently associate with hydrophobic protein regions and prevent protein aggregation. The kinetics of hsp70-substrate binding is governed by the ATP binding and ATPase activity of hsp70 (Flynn et al., 1989). The combination of the peptide and ATP binding functions of hsp70 may be involved in the efficient transfer of antigenic peptides into the MHC class I antigen presentation pathway. Hsp70 also associates with nascent polypeptide chains as they emerge from ribosomes and are involved in stabilizing nascent polypeptides prior to their translocation into various subcellular compartments (Beckmann et al., 1990; Frydman et al., 1994), including chloroplasts, the ER, lysosomes, mitochondria, the nucleus and peroxisomes (Brodsky, 1996; Cyr and Neupert, 1996). The present findings indicate that hsp70 also promotes delivery of covalently linked fusion polypeptides to the subcellular compartment(s) required for cell surface presentation of peptide-MHC-I complexes.

Hsp70's role in intracellular protein breakdown may be especially relevant for the immunogenic effectiveness of its fusion partner. Experiments with yeast cell mutants and with mammalian cell extracts have

shown that, in addition to its function in protein refolding, hsp70 serves an essential role in the degradation of certain abnormal polypeptides (Nelson et al., 1992; Sherman and Goldberg, 1996). Thus, if hsp70 fails to refold a denatured protein, it can facilitate its degradation by the cell's proteolytic machinery. In eukaryotes, hsp70 is essential for the ubiquitination of certain abnormal and regulatory proteins and thus in the breakdown of polyubiquinated polypeptides by the 26S proteasome (Sherman and Goldberg, 1996). The peptides generated by the proteasome in the cytosol appear to be the primary source of the peptides that are translocated into the ER for association with MHC class I. Thus proteins that are linked to hsp70 may be ubiquitinated and processed especially well for presentation with MHC-I proteins.

Immune responses to hsp70 have been detected following exposure to a broad spectrum of infectious agents (Hedstrom et al., 1987; Selkirk et al., 1989; Young et al., 1988). In addition, anti-hsp70 immune responses were induced in infants by the trivalent vaccine against tetanus, diphtheria and pertussis (Del Giudice et al., 1993). It seems that the immune system is routinely stimulated to respond to hsp70 and such stimulation may cause an expansion of hsp70-reactive cells. The cellular responses to mycobacterial hsps are profound; limiting dilution analysis indicates that 20% of the murine $CD4^+$ T lymphocytes that recognize mycobacterial antigens are directed against hsp60 alone (Kaufmann et al., 1987). The high frequency with which human $CD4^+$ T cell clones directed against mycobacterial hsp70 and hsp60 have been detected suggests that these hsps are also major targets of the cellular response in humans (Munk et al., 1988). Thus, although soluble proteins administered in the absence of adjuvant do not typically elicit CD8 CTL, it is possible that the abundant hsp70-reactive helper T cells are

involved in facilitating the unusually efficient CTL response against the soluble hsp70 fusion protein.

Another hsp, gp96, isolated from various tumors and tumor cell lines, has previously been shown to be a potent immunogen for eliciting CD8 CTL. Gp96's effectiveness derives from i) the many peptides that remain bound noncovalently to the protein when isolated from cells (Arnold, et al., 1997; Li and Srivastava, 1993), and ii) its ability to facilitate the transfer of those peptides to MHC-I proteins of "professional" antigen presenting cells (Suto and Srivastava, 1995). Detailed studies of the peptide-binding activity of hsp70 has shown that it has a clear preference for peptides over 7 amino acids in length and those with aliphatic hydrophobic side chains (Flynn, et al., 1991; Rudiger, et al., 1997). Although gp96 can bind many different peptides (Arnold, et al., 1995; Nieland et al., 1996; Udono and Srivastava, 1993), studies with hsp70, as well as general considerations, indicate that no protein can serve as a universal receptor for all peptides. Recombinant hsp70 fusion proteins, in contrast, are thus likely to provide a richer source of peptides available for binding to diverse MHC molecules.

Many different proteins can be linked to hsp70 and the fusion proteins studied so far are effective immunogens in the absence of adjuvants. Hsp70 fusion proteins are thus attractive candidates for vaccines intended to stimulate CD8 CTL in humans.

Materials and Methods

Expression Vector Constructs. The DNA fragment containing the *M. tuberculosis* hsp70 coding sequence was synthesized by PCR using DNA purified from λgt11 clones Y3111 and Y3130 as a template (Young et al., 1987). The complete coding sequence of hsp70 was synthesized by using the upstream primer oKS63 (5'GCCCGGGATCCATGGCTCGTGCGGTCGGGAT3') containing a *BamHI* site immediately before the hsp70 coding sequence and the downstream primer oKS79 (5'GCGGAATTCTCATCAGCCGAGCCGGGGT3') containing an *Eco*RI site immediately after the last coding sequence of hsp70. The DNA fragment containing the ovalbumin coding sequence was synthesized by PCR using plasmid pOv230 (McReynolds et al., 1978) as a template. The upstream primer oKS83 (5'GCGGATCCATATGGTCCTTCAGCCAAGCTCCGTGG3') contained a *Nde*I site immediately before amino acid 161 of ovalbumin and the downstream primer oKS82 (5'GCAGGATCCCTCTTCCATAACATTAGA3') contained a *Bam*HI site immediately after amino acid 276 of ovalbumin. Another downstream primer containing a *Bam*HI site oKS80 (5'GCTGAATTCTTACTCTTCCATAACATTAG3'), included a translation stop codon immediately after amino acid 276 of ovalbumin.

Construction of the vector used to produce hsp70 alone, pKS74, has been previously described (Suzue and Young, 1996). The vector pKS11h was made by modifying the plasmid vector pET11 (Studier et al., 1990) with a histidine tag coding sequence and with the polylinker from pET17b. Plasmid pKS28 was made by subcloning the DNA encoding amino acids 161 to 276 of ovalbumin into the *Nde*I and *Bam*HI sites of pKS11h. Plasmid pKS76 was

created by subcloning ovalbumin (161-276) and hsp70 into the *Nde*I and *Bam*HI sites of pKS11h.

Protein Purification. Cultures of BL21(DE3)pLysS (Studier, et al., 1990) were grown and induced with 0.5 mM isopropylthiogalactoside (IPTG). Hsp70 and ova-hsp70 proteins were both purified as inclusion bodies, refolded stepwise in guanidine and subsequently purified by ATP affinity chromatography as previously described (Suzue and Young, 1996). Protein purity was verified by SDS-PAGE and protein fractions were pooled and dialyzed against PBS. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL).

Peptides. The peptides SIINFEKL (corresponding to ovalbumin amino acids 258-276) and RGYVYQGL (corresponding to the vesicular stomatitis virus nucleoprotein amino acids 324-332), were synthesized by the Biopolymers Facility at the Center for Cancer Research at the Massachusetts Institute of Technology. Peptides were stored as 1 mg/ml stock solutions in PBS.

Mice and Immunizations. 7-8 week old female C57BL/6 mice were obtained from Jackson Laboratories (Bar Harbor, Maine) and Taconic Farms (Germantown, NY). Mice were immunized i.p. on day 0 and s.c. on day 14 with 120 pmoles of purified protein in PBS.

Cell lines. EL4 (H-2^b) thymoma cells, from the American Type Culture Collection (ATCC, Rockville, MD), were grown in RPMI 1640 /10% FCS. E.G7-OVA cells (ovalbumin transfected EL4 cells) (Moore, et al., 1988) were cultured in RPMI 1640 /10% FCS in the presence of 320 μg of G418 per ml. The human cell line T2, is a TAP-deficient, T-B lymphoblastoid fusion hybrid. The K^b transfected clone, T2-K^b, a generous gift from P. Cresswell, was cultured in RPMI 1640 /10% FCS in the presence of 320 μg of G418 per ml.

The CTL clone 4G3 was maintained by weekly restimulation with irradiated E.G7-OVA cells in RPMI 1640/10% FCS/5% rat Con A supernatant (Walden and Eisen, 1990) The C57BL/6-derived melanoma B16 and the ovalbumin-transfected B16 clone, MO5, (Falo et al., 1995) were generously provided by L. Rothstein and L. Sigal. The B16 cells were grown in RPMI 1640 /10% FCS and the MO5 cells were grown in the presence of 2.0 mg of G418 and 40 μg of hygromycin per ml.

IFN-γ ELISA. Spleens were removed from mice 10 days after the last injection. The spleens from 3-10 mice in each treatment group were pooled. Single-cell suspensions were prepared by grinding tissue through a sterile nylon mesh. Erythrocytes were removed by suspending the cells in pH 7.2 lysis buffer (0.15 M NH_4Cl , 1 M $KHCO_3$, 0.1 mM Na_2EDTA) and rinsing the cells two times with RPMI 1640 media. Splenocytes were then cultured at 1 X 10^7 cells/ml in 96-well round bottom microculture plates in RPMI 1640, supplemented with 10% FCS and 50 μM 2-ME at 37°C in 5% CO^2. The cells were stimulated with recombinant ovalbumin (10 μg/ml), SIINFEKL peptide (10 μg/ml), RGYVYQGL (10 μg/ml) or with Con A (5 μg/ml). Cell culture supernatants were removed at 72 h. A sandwich ELISA using paired monoclonal antibodies (Endogen, Cambridge, MA) was used to measure IFN-γ.

CTL assay. Single-cell suspensions of splenocytes were prepared as above. 25 X 10^6 splenocytes were cultured with 5 X 10^6 irradiated (15,000 rads) E.G7-OVA cells in RPMI 1640 supplemented with 10% FCS, 50 μM 2-ME, 1 mM sodium pyruvate and 100 μM non-essential amino acids. After 6-7 days in culture, splenocytes were purified by Ficoll-Paque (Pharmacia, Piscataway, NJ) density centrifugation and then utilized as effector cells.

Target cells were labeled with 100 μCi [^{51}Cr] at 37°C for 1-2 h. For peptide sensitization of target cells, 50 μg of peptide was added to the target cells (300 μg/ml final peptide concentration) during the labeling period. The cells were then rinsed and 5000 [^{51}Cr]-labeled targets and serial dilutions of effector cells were incubated at various E:T ratios in 96 well U-bottom plates at 37°C. For peptide titration assays, the target cells were not pulsed with any peptide during the [^{51}Cr]-labeling period and instead, the peptide was directly added to the 96 well U-bottom plate at final concentrations of 10^{-10} M to 10^{-14} M. Supernatants were harvested after 4-6 h and the radioactivity was measured in a gamma counter. % Specific lysis was calculated as equal to 100 X [(release by CTL-spontaneous release)/(maximal release-spontaneous release)]. Maximal release was determined by addition of 1% Triton X-100 or by resuspending target cells.

In vitro depletion or enrichment of lymphocyte subpopulations. Splenocytes were cultured with irradiated E.G7-OVA cells and purified by Ficoll-Paque (Pharmacia) density centrifugation as described above. Cells were resuspended in cold PBS with 1% FCS and incubated with anti-mouse CD4 (L3T4) microbeads or with anti-mouse CD8a (Ly-2) microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) for 20 min. at 4°C. For cell depletion, the cells were applied on to a Mini MACS column (Miltenyi Biotech) with an attached flow resistor. The cells from the flow-through were collected and used as effector cell in the cytolytic assay. For positive selection of CD8 cells, the cells were applied on to a Mini MACS column without a flow resistor. The column was rinsed and the cells adhering to the column were released by removing the column from the magnetic holder. The positively selected cells were then used as effector cells in the cytolytic assay. The effectiveness of positive and negative selection of cells was verified by flow cytometry using

phycoerythrin conjugated anti-mouse CD4 and fluorescein isothiocyanate conjugated anti-mouse CD8a antibodies (Pharmingen, San Diego, CA).

Tumor protection assay. C57BL/6 mice were injected i.p. with 120 pmoles of ova or ova-hsp70 without adjuvant and boosted s.c. 2 weeks later. Ten days after the last immunization the mice were injected s.c. on the right flank with 1 X 10^5 MO5 tumor cells or with 1 X 10^5 B16 tumor cells. As a control, unimmunized mice were also inoculated with the tumor cells. 5 to 10 mice were used for each experimental group. On the day of the tumor challenge, the B16 and MO5 cells were harvested by trypsinization and rinsed three times in PBS. The cells were resuspended in PBS and administered s.c. in a volume of 0.1 ml. Tumor growth was assessed by measuring the diameter of the tumor in millimeters (recorded as the average of two perpendicular diameter measurements). Mice that became moribund were sacrificed. Consistent results were observed in three separate experiments.

Acknowledgments

We thank James Forman and Hidde Ploegh for helpful reviews of the manuscript. We thank Luis Sigal and Lisa Rothstein for generously providing the cell lines B16 and MO5 and we thank Glen Paradis for assistance with the FACS analysis. The excellent technical assistance of Carol McKinley is gratefully acknowledged. This work was supported by the National Institutes of Health Grants AI26463 and AI31869.

Section III References

Albert, M. L., B. Sauter and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature* 392:86-89.

Andersen, P. and I. Heron. 1993. Specificity of a protective memory immune response against Mycobacterium tuberculosis. *Infect Immun* 61:844-51.

Androlewicz, M. J., K. S. Anderson and P. Cresswell. 1993. Evidence that transporters associated with antigen processing translocate a major histocompatibility complex class I-binding peptide into the endoplasmic reticulum in an ATP-dependent manner. *Proc Natl Acad Sci U S A* 90:9130-4.

Arnold, D., S. Faath, H. Rammensee and H. Schild. 1995. Cross-priming of minor histocompatibility antigen-specific cytotoxic T cells upon immunization with the heat shock protein gp96. *J Exp Med* 182:885-889.

Arnold, D., C. Wahl, S. Faath, H. Rammensee and H. Schild. 1997. Influences of transporter associated with antigen processing (TAP) on the repertoire of peptides associated with the endoplasmic reticulum-resident stress protein gp96. *J Exp Med* 186:461-466.

Bachmann, M. F., A. Oxenius, H. Pircher, H. Hengartner, P. A. Ashton-Richardt, S. Tonegawa and R. M. Zinkernagel. 1995. TAP1-independent loading of class I molecules by exogenous viral proteins. *Eur J Immunol* 25:1739-43.

Beckmann, R. P., L. E. Mizzen and W. J. Welch. 1990. Interaction of Hsp 70 with newly synthesized proteins: implications for protein folding and assembly. *Science* 248:850-4.

Bevan, M. J. 1976. Cross-priming for a secondary cytotoxic response to minor H antigens with H-2 congenic cells which do not cross-react in the cytotoxic assay. *J Exp Med* 143:1283-1288.

Bohm, W., R. Schirmbeck, A. Elbe, K. Melber, D. Diminky, G. Kraal, N. van Rooijen, Y. Barenholz and J. Reimann. 1995. Exogenous hepatitis B surface antigen particles processed by dendritic cells or macrophages prime murine MHC class I restricted cytotoxic T lymphocytes in vivo. *J Immunology* 155:3313-3321.

Braciale, T. J., L. A. Morrison, M. T. Sweetser, J. Sambrook, M. J. Gething and V. L. Braciale. 1987. Antigen presentation pathways to class I and class II MHC-restricted T lymphocytes. *Immunol Rev* 98:95-114.

Brodsky, F. M. and L. E. Guagliardi. 1991. The cell biology of antigen processing and presentation. *Annu Rev Immunol* 9:707-44.

Brodsky, J. L. 1996. Post-translational protein translocation: not all hsc70s are created equal. *Trends Biochem Sci* 21:122-6.

Byrne, J. A. and M. B. Oldstone. 1984. Biology of cloned cytotoxic T lymphocytes specific for lymphocytic choriomeningitis virus: clearance of virus in vivo. *J Virol* 51:682-6.

Carbone, F. R. and M. J. Bevan. 1989. Induction of ovalbumin-specific cytotoxic T cells by in vivo peptide immunization. *J Exp Med* 169:603-12.

Carbone, F. R. and M. J. Bevan. 1990. Class I-restricted processing and presentation of exogenous cell-associated antigen in vivo. *J Exp Med* 171:377-387.

Chapman, H. A. 1998. Endosomal proteolysis and MHC class II function. *Current Opinion in Immunology* 10:93-102.

Cyr, D. M. and W. Neupert. 1996. Roles for hsp70 in protein translocation across membranes of organelles. In *Stress-Inducible Cellular Responses*, U. Feige, R. I. Morimoto, I. Yahara and B. S. Polla, eds. Birkhauser Verlag, Basel, p. 25-40.

Del Giudice, G., A. Gervaix, P. Costantino, C. A. Wyler, C. Tougne, E. R. de Graeff-Meeder, J. Van Embden, R. Van der Zee, L. Nencioni, R. Rappuoli, S. Suter and P. H. Lambert. 1993. Priming to heat shock proteins in infants vaccinated against pertussis. *J Immunol* 150:2025-32.

Driscoll, J. and D. Finley. 1992. A controlled breakdown: antigen processing and the turnover of viral proteins. *Cell* 68:823-5.

Falo, L., Jr., M. Kovacsovics-Bankowski, K. Thompson and K. L. Rock. 1995. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. *Nat Med* 1:649-653.

Flynn, G. C., T. G. Chappell and J. E. Rothman. 1989. Peptide binding and release by proteins implicated as catalysts of protein assembly. *Science* 245:385-90.

Flynn, G. C., J. Pohl, M. T. Flocco and J. E. Rothman. 1991. Peptide-binding specificity of the molecular chaperone BiP. *Nature* 353:726-30.

Frydman, J., E. Nimmesgern, K. Ohtsuka and F. U. Hartl. 1994. Folding of nascent polypeptide chains in a high molecular mass assembly with molecular chaperones. *Nature* 370:111-7.

Gething, M. J. and J. Sambrook. 1992. Protein folding in the cell. *Nature* 355:33-45.

Goldberg, A. L. and K. L. Rock. 1992. Proteolysis, proteasomes and antigen presentation. *Nature* 357:375-9.

Harding, C. 1992. Electroporation of exogenous antigen into the cytosol for antigen processing and class I major histocompatibility complex (MHC) presentation: weak base amines and hypothermia (18 degrees C) inhibit the class I MHC processing pathway. *Eur J Immunol* 22:1865-9.

Harding, C. V. and R. Song. 1994. Phagocytic processing of exogenous particulate antigens by macrophages for presentation by class I MHC molecules. *J Immunol* 153:4925-33.

Hartl, F. U. 1996. Molecular chaperones in cellular protein folding. *Nature* 381:571-9.

Hartl, F. U., R. Hlodan and T. Langer. 1994. Molecular chaperones in protein folding: the art of avoiding sticky situations. *Trends Biochem Sci* 19:20-5.

Hedstrom, R., J. Culpepper, R. A. Harrison, N. Agabian and G. Newport. 1987. A major immunogen in Schistosoma mansoni infections is homologous to the heat-shock protein Hsp70. *J Exp Med* 165:1430-5.

Heemels, M. T. and H. Ploegh. 1995. Generation, translocation, and presentation of MHC class I-restricted peptides. *Annu Rev Biochem* 64:463-491.

Huang, A. Y., A. T. Bruce, D. M. Pardoll and H. I. Levitsky. 1996. In vivo cross-priming of MHC class I-restricted antigens requires the TAP transporter. *Immunity* 4:349-55.

Jondal, M., R. Schirmbeck and J. Reimann. 1996. MHC class I-restricted CTL responses to exogenous antigens. *Immunity* 5:295-302.

Kast, W. M., A. M. Bronkhorst, L. P. de Waal and C. J. Melief. 1986. Cooperation between cytotoxic and helper T lymphocytes in protection against lethal Sendai virus infection. Protection by T cells is MHC-restricted and MHC-regulated; a model for MHC-disease associations. *J Exp Med* 164:723-38.

Kaufmann, S. H., U. Vath, J. E. Thole, J. D. Van Embden and F. Emmrich. 1987. Enumeration of T cells reactive with Mycobacterium tuberculosis organisms and specific for the recombinant mycobacterial 64-kDa protein. *Eur J Immunol* 17:351-7.

Ke, Y., Y. Li and J. A. Kapp. 1995. Ovalbumin injected with complete Freund's adjuvant stimulates cytolytic responses. *Eur J Immunol* 25:549-553.

Kovacsovics-Bankowski, M., K. Clark, B. Benacerraf and K. L. Rock. 1993. Efficient major histocompatibility complex class I presentation of exogenous antigen upon phagocytosis by macrophages. *Proc Natl Acad Sci U S A* 90:4942-6.

Kovacsovics-Bankowski, M. and K. L. Rock. 1995. A phagosome-to-cytosol pathway for exogenous antigens presented on MHC class I molecules. *Science* 267:243-246.

Li, Z. and P. K. Srivastava. 1993. Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation. *Embo J* 12:3143-51.

Lin, Y. L. and B. A. Askonas. 1981. Biological properties of an influenza A virus-specific killer T cell clone. Inhibition of virus replication in vivo and induction of delayed-type hypersensitivity reactions. *J Exp Med* 154:225-34.

Lukacher, A. E., V. L. Braciale and T. J. Braciale. 1984. In vivo effector function of influenza virus-specific cytotoxic T lymphocyte clones is highly specific. *J Exp Med* 160:814-26.

Martinez-Kinader, B., G. B. Lipford, H. Wagner and K. Heeg. 1995. Sensitization of MHC class I-restricted T cells to exogenous proteins: evidence for an alternative class I-restricted antigen presentation pathway. *Immunology* 86:287-295.

McReynolds, L., B. W. O'Malley, A. D. Nisbet, J. E. Fothergill, D. Givol, S. Fields, M. Robertson and G. G. Brownlee. 1978. Sequence of chicken ovalbumin mRNA. *Nature* 273:723-8.

Moore, M. W., F. R. Carbone and M. J. Bevan. 1988. Introduction of soluble protein into the class I pathway of antigen processing and presentation. *Cell* 54:777-785.

Morrison, L. A., A. E. Lukacher, V. L. Braciale, D. P. Fan and T. J. Braciale. 1986. Differences in antigen presentation to MHC class I-and class II-restricted influenza virus-specific cytolytic T lymphocyte clones. *J Exp Med* 163:903-21.

Munk, M. E., B. Schoel and S. H. Kaufmann. 1988. T cell responses of normal individuals towards recombinant protein antigens of Mycobacterium tuberculosis. *Eur J Immunol* 18:1835-8.

Nagler-Anderson, C., C. R. Verret, A. A. Firmenich, M. Berne and H. N. Eisen. 1988. Resistance of primary CD8+ cytotoxic T lymphocytes to lysis by cytotoxic granules from cloned T cell lines. *J Immunol* 141:3299-305.

Neefjes, J. J., F. Momburg and G. J. Hammerling. 1993. Selective and ATP-dependent translocation of peptides by the MHC-encoded transporter. *Science* 261:769-71.

Nelson, R. J., T. Ziegelhoffer, C. Nicolet, M. Werner-Washburne and E. A. Craig. 1992. The translation machinery and 70 kd heat shock protein cooperate in protein synthesis. *Cell* 71:97-105.

Newman, M. J., J. Y. Wu, B. H. Gardner, K. J. Munroe, D. Leombruno, J. Recchia, C. R. Kensil and R. T. Coughlin. 1992. Saponin adjuvant induction of ovalbumin-specific CD8+ cytotoxic T lymphocyte responses. *J Immunol* 148:2357-62.

Nieland, T. J., M. C. Tan, M. Monne-van Muijen, F. Koning, A. M. Kruisbeek and G. M. van Bleek. 1996. Isolation of an immunodominant viral peptide that is endogenously bound to the stress protein GP96/GRP94. *Proc Natl Acad Sci U S A* 93:6135-9.

Norbury, C. C., B. J. Chambers, A. R. Prescott, H. Ljunggren and C. Watts. 1997. Constitutive macropinocytosis allows TAP-dependent major histocompatibility complex class I presentation of exogenous soluble antigen by bone marrow-derived dendritic cells. *Eur J Immunol* 27:280-288.

Norbury, C. C., L. J. Hewlett, A. R. Prescott, N. Shastri and C. Watts. 1995. Class I MHC presentation of exogenous soluble antigen via macropinocytosis in bone marrow macrophage. *Immunity* 3:783-791.

Pfeifer, J. D., M. J. Wick, R. L. Roberts, K. Findlay, S. J. Normark and C. V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature* 361:359-62.

Raychaudhuri, S., M. Tonks, F. Carbone, T. Ryskamp, W. J. Morrow and N. Hanna. 1992. Induction of antigen-specific class I-restricted cytotoxic T cells by soluble proteins in vivo. *Proc Natl Acad Sci U S A* 89:8308-8312.

Reis e Sousa, C. and R. N. Germain. 1995. Major histocompatibility complex class I presentation of peptides derived from soluble exogenous antigen by a subset of cells engaged in phagocytosis. *J Exp Med* 182:841-51.

Rock, K. L. 1996. A new foreign policy: MHC class I molecules monitor the outside world. *Immunology Today* 17:131-137.

Rock, K. L., L. Rothstein, S. Gamble and C. Fleischacker. 1993. Characterization of antigen-presenting cells that present exogenous antigens in association with class I MHC molecules. *J Immunol* 150:438-446.

Rotzschke, O., K. Falk, S. Stevanovic, G. Jung, P. Walden and H. G. Rammensee. 1991. Exact prediction of a natural T cell epitope. *Eur J Immunol* 21:2891-2894.

Rudiger, S., L. Germeroth, J. Schneider-Mergener and B. Bukau. 1997. Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *Embo J* 16:1501-7.

Schirmbeck, R., W. Bohm and J. Reimann. 1994a. Injection of detergent-denatured ovalbumin primes murine class I-restricted cytotoxic T cells in vivo. *Eur J Immunol* 24:2068-2072.

Schirmbeck, R., K. Melber and J. Reimann. 1995. Hepatitis B virus small surface antigen particles are processed in a novel endosomal pathway for major histocompatibility complex class I-restriceted epitope presentation. *Eur J Immunol* 25:1063-1070.

Selkirk, M. E., D. A. Denham, F. Partono and R. M. Maizels. 1989. Heat shock cognate 70 is a prominent immunogen in Brugian filariasis. *J Immunol* 143:299-308.

Shepherd, J. C., T. N. Schumacher, P. G. Ashton-Rickardt, S. Imaeda, H. L. Ploegh, C. Janeway Jr. and S. Tonegawa. 1993. TAP1-dependent peptide translocation in vitro is ATP dependent and peptide selective. *Cell* 74:577-84.

Sherman, M. Y. and A. L. Goldberg. 1996. Involvement of molecular chaperones in intracellular protein breakdown. In *Stress-Inducible Cellular Responses*, U. Feige, R. I. Morimoto, I. Yahara and B. S. Polla, eds. Birkhauser Verlag, Basel, p. 57-78.

Studier, F. W., A. H. Rosenberg, J. J. Dunn and J. W. Dubendorff. 1990. Use of T7 RNA polymerase to direct expression of cloned genes. *Methods Enzymol* 185:60-89.

Suto, R. and P. K. Srivastava. 1995. A mechanism for the specific immunogenicity of heat shock protein-chaperoned peptides. *Science* 269:1585-8.

Suzue, K. and R. A. Young. 1996. Adjuvant-free hsp70 fusion protein system elicits humoral and cellular immune responses to HIV-1 p24. *J Immunol* 156:873-9.

Townsend, A. and H. Bodmer. 1989. Antigen recognition by class I-restricted T lymphocytes. *Annu Rev Immunol* 7:601-24.

Townsend, S. E. and J. P. Allison. 1993. Tumor rejection after direct costimulation of CD8+ T cells by B7-transfected melanoma cells. *Science* 259:368-70.

Udono, H. and P. K. Srivastava. 1993. Heat shock protein 70-associated peptides elicit specific cancer immunity. *J Exp Med* 178:1391-6.

Udono, H. and P. K. Srivastava. 1994. Comparison of tumor-specific immunogenicities of stress-induced proteins gp96, hsp90, and hsp70. *J Immunol* 152:5398-403.

Van Bleek, G. M. and S. G. Nathenson. 1990. Isolation of an endogenously processed immunodominant viral peptide from the class I H-2Kb molecule. *Nature* 348:213-216.

Walden, P. R. and H. N. Eisen. 1990. Cognate peptides induce self-destruction of CD8+ cytolytic T lymphocytes. *Proc Natl Acad Sci U S A* 87:9015-9019.

Wijburg, O. L. C., G. van den Dobbelsteen, J. Vadolas, A. Sanders, R. A. Strugnell and N. van Rooijen. 1998. The role of macrophages in the induction and regulation of immunity elicited by exogenous antigens. *Eur J Immunol* 28:479-487.

Wolf, P. R. and H. L. Ploegh. 1995. How MHC class II molecules acquire their peptide cargo: Biosynthesis and trafficking through the endocytic pathway. *Ann Rev Cell Dev Biol* 11:267-306.

Xu, X. and S. K. Pierce. 1995. The novelty of antigen-processing compartments. *J Immunol* 1652-1654.

Yewdell, J. W., J. R. Bennink and Y. Hosaka. 1988. Cells process exogenous proteins for recognition by cytotoxic T lymphocytes. *Science* 239:637-40.

Young, D., R. Lathigra, R. Hendrix, D. Sweetser and R. A. Young. 1988. Stress proteins are immune targets in leprosy and tuberculosis. *Proc Natl Acad Sci U S A* 85:4267-70.

Young, D. B., L. Kent and R. A. Young. 1987. Screening of a recombinant mycobacterial DNA library with polyclonal antiserum and molecular weight analysis of expressed antigens. *Infect Immun* 55:1421-5.

Zhu, X., X. Zhao, W. F. Burkholder, A. Gragerov, C. M. Ogata, M. E. Gottesman and W. A. Hendrickson. 1996. Structural analysis of substrate binding by the molecular chaperone DnaK. *Science* 272:1606-1614.

Section IV

Further Experimental Studies

Section IV: Further Experimental Studies.

Section one discussed the chaperoning functions and the immunogenic nature of hsp70. In section II, immunological carrier proteins and adjuvants were presented and included data illustrating the effectiveness of hsp70 as an adjuvant-free carrier to elicit humoral responses to an attached antigen. Section III described my work which demonstrates that an hsp70 fusion protein administered in the absence of adjuvant can elicit CTLs and induce protective immunity in mice. How does hsp70 assist in the elicitation of humoral and cellular responses? Currently, experiments are ongoing in the laboratory to address this question. This last section of the thesis contains unpublished experimental results, which provide some insight to the mechanism by which hsp70 functions as an immnological carrier.

Is the ATP-binding domain or the peptide binding domain of hsp70 sufficient for the adjuvant-free carrier effect?

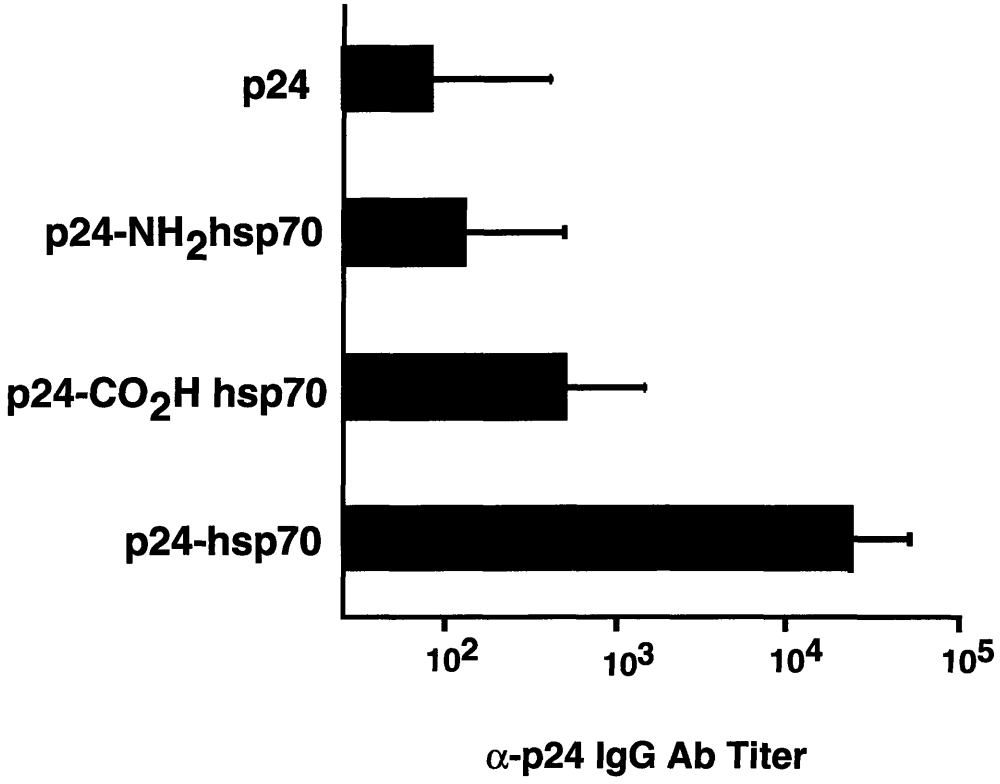
Humoral response-

I investigated whether the peptide binding or the ATP binding domain of hsp70 was sufficient for eliciting antibody responses to the attached p24 antigen. As discussed in chapter 1, the amino terminal 44 kD portion of hsp70 contains the ATP binding domain, and the carboxyl terminal portion of hsp70 binds polypeptide substrates. Recombinant fusion proteins were produced with the ATP binding domain of hsp70 attached to p24 (p24-NH_2 hsp70) and the peptide binding domain of hsp70 attached to p24 (p24-CO_2H hsp70). These proteins were purified from *E. coli* as inclusion bodies, refolded and purified using NTA-Ni^{2+} chromatography.

I examined the ability of the p24 fusion proteins to elicit anti-p24 antibody responses in the absence of adjuvant. Groups of BALB/c mice were inoculated with 50 pmoles of one of the following proteins: p24, p24-NH_2 hsp70, p24-CO_2H, or with p24 fused to the whole hsp70 molecule (p24-hsp70). A second equivalent dose was given at three weeks. Serum samples were obtained three weeks after the second immunization and anti-p24 IgG antibody titers were determined by ELISA. Mice injected with p24-hsp70 had high levels of anti-p24 antibody (Fig. 1). In comparison, mice inoculated with p24-NH_2 hsp70 or with p24-CO_2H had anti-p24 antibody levels which were only two or four-fold higher than that elicited by the administration of p24 alone.

Figure 1

Anti-p24 antibody response of BALB/c mice after administration of p24-hsp70 fusion proteins



This experiment demonstrates that administration of HIV-1 p24 fusion proteins containing portions of hsp70 does not elicit anti-p24 humoral responses very effectively. This result suggested to me that perhaps the chaperoning function of hsp70 was necessary for the adjuvant-free carrier effect of hsp70. At that time, I was beginning studies on the use of hsp70 fusion proteins to elicit CTL responses (the work described in chapter 6). Thus, I decided make hsp70 fusion proteins with ovalbumin due to the availability of reagents for studying CTL responses against ovalbumin and the hsp70 molecules attached to ovalbumin were defective in chaperoning function.

Can mutant hsp70 molecules function as adjuvant-free carriers?

Examination of CTL responses

The amino acid point mutations in DnaK of glutamic acid at position 171 to lysine (E171K) and of glycine at position 229 to aspartic acid (G229D) confer a temperature-sensitive phenotype in *E. coli* (Wild et al., 1992). These are highly conserved residues (Figure 2A) in the ATP binding domain of hsp70. The E171K mutation results in defective ATPase activity and the G229D mutation results in defective ATP-binding. Hsp70 binds to hydrophobic peptide segments in an ATP-dependent manner and thus the E171K and 229D mutations interfere with the chaperoning function of hsp70 (Gaut and Hendershot, 1993; Liberek et al., 1991). The corresponding mutations in mycobacterial hsp70, glycine at position 201 and glutamic acid at position 147 (Fig. 2A), were made by PCR and the mutant proteins were expressed as ovalbumin-hsp70 fusion proteins. The fusion proteins (ova-hsp70 G201D, ova-hsp70 E147K) were purified from *E. coli* as inclusion bodies, refolded and purified using NTA-Ni^{2+} chromatography.

In order to assess whether the mutant hsp70 molecules could function as adjuvant-free carriers, mice were injected i.p. with 120 pmoles of ova, ova-hsp70 WT, ova-hsp70 G201D or ova-hsp70 E147K in saline. The injections were repeated s.c. 2 weeks later. Mice were sacrificed 10 days after the boost and for each mouse group, 5-10 spleens were pooled and splenocytes from immunized mice were incubated for 6 days in the presence of irradiated E.G7-OVA cells without added interleukins. The cultured cells were then used as effector cells in CTL assays. Cells from mice injected with ovalbumin protein were unable to lyse EL4 target cells or E.G7-OVA cells (Figure 2B). In contrast, effector cells from mice primed with ovalbumin-hsp70 WT, ova-hsp70 G201D or ova-hsp70 E147K fusion protein were able to lyse E.G7-OVA cells. Thus, the point mutations which affected the ATP binding and hydrolysis functions of hsp70 had no observable effect on the ability of hsp70 to assist in the induction of CTL responses against an attached antigen.

Figure 2

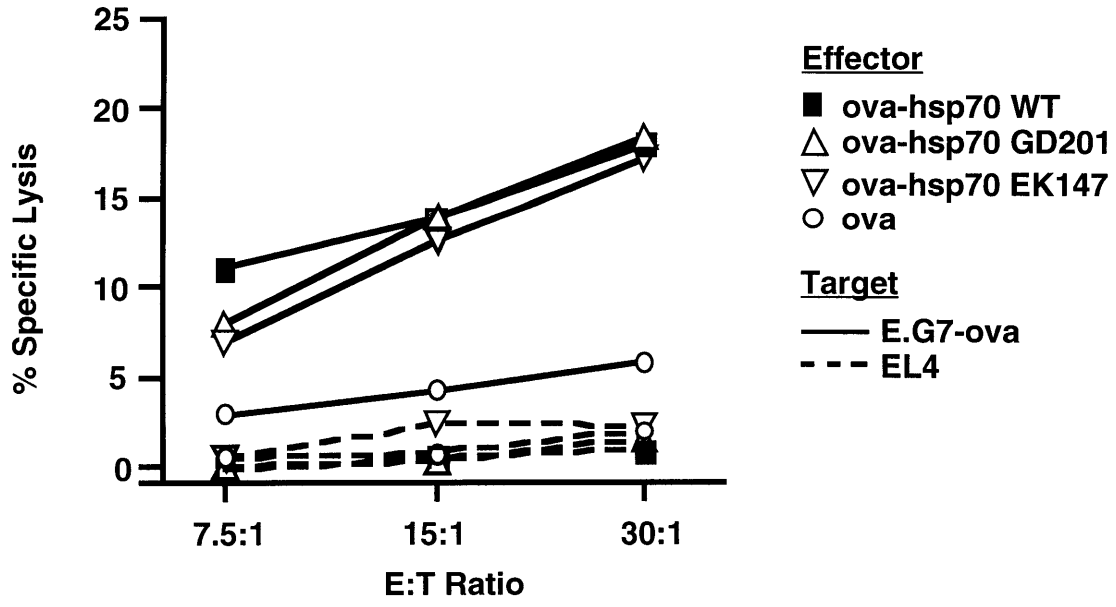
A **Hsp70 ATPase/ATP binding mutations**

137	AGLNVLRI VNEPTAAAPGYGLDK- GEKEQ	*M. tuberculosis* hsp70
161	AGLEVKRI I NEPTAAALAYGLDK- GTGNR	*E. coli* DnaK
196	AGLNVVRI I NEPTAAAI AYGLDKKD- GER	*C. elegans* BiP
186	AGLNVI RVVNEPTAAALAYGMDK- TK- DS	*L. major* hsp70.1
212	SGLNVLRVI NEPTAAALAYGLDK- SE- DK	Murine Pbp74
165	AGLNVLRI I NEPTAAAI AYGLDRTGKGER	Human hsp70-2

165	RI LVFDLGGGTFDVSLLEI- - - - GEGVVE	*M. tuberculosis* hsp70
189	TI AVYDLGGGTFDI SI I EI DEVDGEKTFE	*E. coli* DnaK
224	NI LVFDLGGGTFDVSMLTI DNG- - - - VFE	*C. elegans* BiP
213	LI AVYDLGGGTFDI SVLEI AGG- - - - VFE	*L. major* hsp70.1
239	VI AVYDLGGGTFDI SI LEI QKG- - - - VFE	Murine Pbp74
194	NVLI FDLGGGTFDVSI LTI DDG- - - - I FE	Human hsp70-2

190	VRATSGDNHLGGDDWDQRVVDWLVDKFKG	*M. tuberculosis* hsp70
218	VLATNGDTHLGGEDFDSRLI NYLVEEFKK	*E. coli* DnaK
249	VLATNGDTHLGGEDFDQRVMEYFI KLYKK	*C. elegans* BiP
238	VKATNGDTHLGGEDFDLALSDYI LEEFRK	*L. major* hsp70.1
264	VKSTNGDTFLGGEDFDQALLRHI VKEFKR	Murine Pbp74
219	VKATAGDTHLGGEDFDNRLVNHFVEEFKR	Human hsp70-2

B **Mutant ova-hsp70 fusion proteins elicit anti-ova CTL**

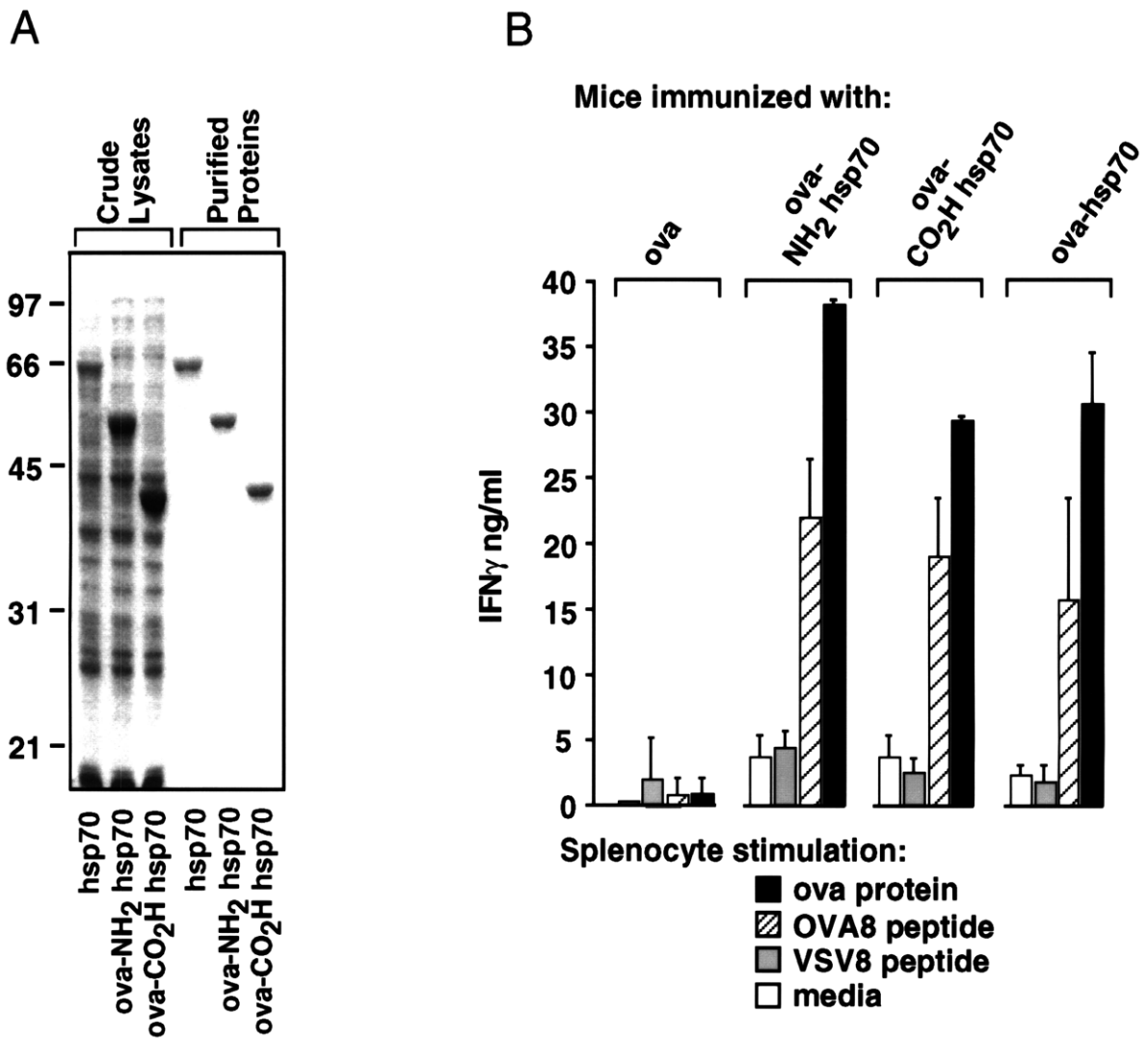


Is the ATP-binding domain or the peptide binding domain of hsp70 sufficient for the adjuvant-free carrier effect?

Cellular responses-

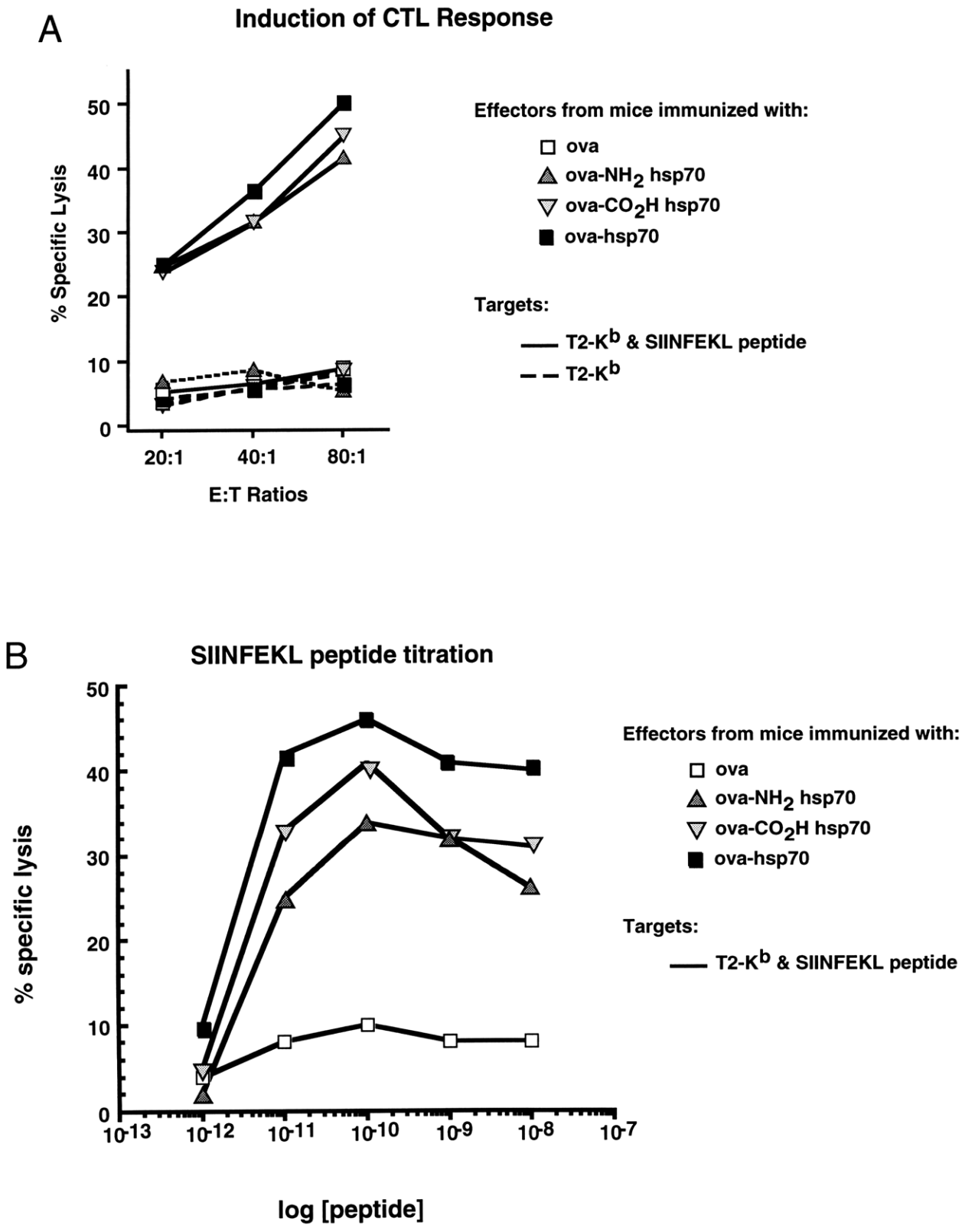
Since the chaperoning function of hsp70 did not appear to be essential for the delivery of ovalbumin into the MHC class I presentation pathway, I investigated whether the peptide binding or the ATP binding domain of hsp70 was sufficient for eliciting T cell responses to the attached ovalbumin antigen. Although either domain of hsp70 was sufficient for the adjuvant-free carrier effect of hsp70 in the induction of the humoral response, it was possible that hsp70 functioned via a different mechanism to induce cellular responses to an attached antigen. Recombinant fusion proteins were produced with the ATP binding domain of hsp70 attached to ovalbumin (ovalbumin-NH_2 hsp70) and the peptide binding domain of hsp70 attached to ovalbumin (ovalbumin-CO_2H hsp70). These proteins were purified from *E. coli* as inclusion bodies, refolded and purified using NTA-Ni^{2+} chromatography (Figure 3A).

Splenocyte T cell responses to ovalbumin were assessed after injecting mice with ovalbumin-NH_2 hsp70 or with ovalbumin-CO_2H hsp70 fusion protein in saline solution (Fig. 3B and Fig. 4). The anti-ovalbumin T cell response in the immunized mice were examined by stimulating splenocytes in culture with the SIINFEKL peptide or with the ovalbumin protein antigen. Similar levels of IFN-γ was released from the ova-hsp70, ova-NH_2 hsp70 or the ova-CO_2H hsp70 groups (Fig. 3B). The release of IFN-γ was ovalbumin-specific, since splenocytes cultured in media alone or with control RGYVYQGL peptide secreted low levels of IFN-γ.


A
Crude Lysates
Purified Proteins
97
66
45
31
21
hsp70
ova-NH2 hsp70
ova-CO2H hsp70
hsp70
ova-NH2 hsp70
ova-CO2H hsp70
B
Mice immunized with:
ova
ova-NH2 hsp70
ova-CO2H hsp70
ova-hsp70
IFNγ ng/ml
40
35
30
25
20
15
10
5
0
Splenocyte stimulation:
ova protein
OVA8 peptide
VSV8 peptide
media

In the cytolytic assay, the effector cells from ova-NH_2 hsp70 or the ova-CO_2H hsp70 groups were able to effectively lyse E.G7-OVA target cells but not the EL4 control cells (Fig. 4A). As described in chapter 6, the effectiveness of the CTL in immunized mice were examined by using T2-K^b as target cells and SIINFEKL at various concentrations. As shown in Fig. 4B, half-maximal lysis was obtained for the ova-hsp70, ova-NH_2 hsp70 and ova-CO_2H hsp70 groups at about the same peptide concentration. (It may be noted that the peptide concentration at which half-maximal lysis was obtained for the ova-hsp70 group in Fig. 4B in this chapter differs from that in Fig. 1B in chapter 6. This is due to the different length of time which the SIINFEKL peptide was pre-incubated with the T2-K^b target cells.) The peptide titration assay demonstrates that CTL from the ova-NH_2 hsp70 and ova-CO_2H hsp70 immunized mice were equally effective as the CTL from the ova-hsp70-immunized mice in terms of the SIINFEKL concentration required for half-maximal lysis. Thus, administering soluble protein with either the amino or the carboxyl terminal portion of hsp70 fused to ovalbumin is sufficient to elicit anti-ovalbumin T cell responses.

Figure 4


A
Induction of CTL Response
% Specific Lysis
50
40
30
20
10
0
20:1
40:1
80:1
E:T Ratios
Effectors from mice immunized with:
ova
ova-NH2 hsp70
ova-CO2H hsp70
ova-hsp70
Targets:
T2-Kb & SIINFEKL peptide
T2-Kb
B
SIINFEKL peptide titration
% specific lysis
50
40
30
20
10
0
10-13
10-12
10-11
10-10
10-9
10-8
10-7
log [peptide]
Effectors from mice immunized with:
ova
ova-NH2 hsp70
ova-CO2H hsp70
ova-hsp70
Targets:
T2-Kb & SIINFEKL peptide

Concluding Remarks

The experimental results currently suggest a different mechanism of action for hsp70 in the induction of humoral versus cellular immune responses. However, the humoral responses described in Section IV were assessed with the HIV p24 antigen while the cellular immune responses were examined with ovalbumin as the antigen of interest. It is possible that there is an antigen specific variable and the conditions necessary to elicit immune responses against p24 in comparison to ovalbumin may differ. The humoral response against ovalbumin should be examined with the various ova-hsp70 fusion proteins. In order to examine the CTL response against p24, the appropriate p24 target cell line is in the process of being made. Are there in fact specific portions of hsp70 which are sufficient for the adjuvant-free carrier effect? Are there particular T cell epitopes of hsp70 which are essential? Is the interaction of hsp70-fusion proteins with other membrane proteins involved? Do the hsp70 fusion proteins have an enhanced ability to enter into cellular compartments? Do antigen presenting cells such as dendritic cells or macrophages present an antigen in the context of MHC class I molecules more efficiently as an hsp70 fusion protein? These are the types of issues which will continue being addressed in the future.

Section IV References

Gaut, J. R. and L. M. Hendershot. 1993. Mutations within the nucleotide binding site of immunoglobulin-binding protein inhibit ATPase activity and interfere with release of immunoglobulin heavy chain. *J Biol Chem* 268:7248-7255.

Liberek, K., D. Skowyra, M. Zylicz, C. Johnson and C. Georgopoulos. 1991. The *Escherichia coli* DnaK chaperone, the 70-kDa heat shock protein eukaryotic equivalent, changes conformation upon ATP hydrolysis, thus triggering its dissociation from a bound target protein. *J Biol Chem* 266:14491-14496.

Wild, J., A. Kamath-Loeb, E. Ziegelhoffer, M. Lonetto, Y. Kawasaki and C. A. Gross. 1992. Partial loss of function mutations in DnaK, the Escherichia coli homologue of the 70-kDa heat shock proteins, affect highly conserved amino acids implicated in ATP binding and hydrolysis. *Proc Natl Acad Sci U S A* 89:7139-7143.