Order Number 9405550

From tumor rejection antigen to protein chaperone: Exploration of the biochemical basis of tumor-specific immunogenicity of heat shock protein gp96

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City University of New York, 1993

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FROM TUMOR REJECTION ANTIGEN TO PROTEIN CHAPERONE: EXPLORATION OF THE BIOCHEMICAL BASIS OF TUMOR-SPECIFIC IMMUNOGENICITY OF HEAT SHOCK PROTEIN GP96

by

ZIHAI LI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

1993

This manuscript has been read and accepted for the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

July 27, 1993
Date

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Abstract

FROM TUMOR REJECTION ANTIGEN TO PROTEIN CHAPERONE: EXPLORATION OF THE BIOCHEMICAL BASIS OF TUMOR-SPECIFIC IMMUNOGENICITY OF HEAT SHOCK PROTEIN GP96

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ZIHAI LI

Advisor: Professor Pramod K. Srivastava

Immunogenicity of tumors was first demonstrated convincingly in chemically-induced sarcomas of inbred mice. A search for the molecules which mediate this immunity led to the identification of heat shock protein (HSP) gp96 as tumor rejection antigen in methylcholanthrene-induced sarcomas of inbred BALB/c mice. Since gp96 is present in normal tissues as well, it was suggested previously that there were mutations in gp96 genes of tumors. However, sequencing of gp96 cDNAs from tumors as well as normal tissues did not reveal any tumor-specific mutations. These observations raise a question as to the structural basis of tumor-specific immunogenicity of gp96.

As gp96 is a HSP and HSPs are known for their ability to bind a wide array of molecules including peptides, a hypothesis is proposed that gp96 may not be immunogenic *per se*, but may be a carrier of antigenic peptides. In view of the predominant localization of gp96 in the lumen of the ER, it is further suggested that gp96 acts as a peptide-acceptor in the ER and enables peptide-loading of MHC class I molecules.

Some elements of these hypotheses were tested in the present work. The major observations are: 1) gp96 is a Mg^{2+}-dependent ATPase. The activity has a Km of 8 μM and the turn over rate of 0.08 mole/min/mole; 2) The ATPase activity of gp96 is stimulated by

misfolded proteins such as dephosphorylated casein but not by peptides; 3) Gp96 associates with MHC class I in human T2 cells as well as murine RMA-S and EL-4 cells; 4) Immunoprecipitation by conformation-dependent antibodies against MHC class I indicates that gp96 binds preferentially unfolded and thus peptide-free MHC class I; 5) Peptides can be extracted from purified gp96, a property of gp96 similar to that of the MHC molecules.

Based on the above results, I propose that gp96 is a protein chaperone for peptides and MHC class I. A model is presented to explain the role of gp96 in the peptide-loading step of the folding/assembly of MHC class I.

ACKNOWLEDGMENT

It is the love, support and encouragement from my family that has made it possible for me to pursue a scientific career. This dissertation is thus dedicated to them including my parents, my wife Shaoli and my daughter Janet.

I wish to express my gratitude to Drs. Lu-Hai Wang and Miriam Siekevitz for their patience and generosity in teaching me molecular biology techniques during my first two laboratory rotations. I thank Dr. Peter Palese for his consistent inspiration and encouragement as I am trained in the Department of Microbiology. Moreover, I am indebted to Drs. Alan Frey, Lloyd Mayer, Karen Zier, Lu-Hai Wang and Jerome Schulman for their valuable suggestions during the course of my work. I am also grateful to Dr. Jack Peter Green for allowing me to use the facilities of the Department of Pharmacology. And of course, I owe a debt of gratitude to all the members of our laboratory, Heiichiro Udono, Nathalie Blachere, Anne Altmeyer, Sylvia Janetzki and Daniel Levey to offer help and discussions in all the experiments described.

Finally, my deepest gratitude is due to Dr. Pramod Srivastava for his great wisdom and patience to guide me through this project; his generosity to allow me to share with him his profound knowledge and skills in cancer immunology as well as his inspiration for my determination to devote my life to biomedical science.

PREFACE

This dissertation was written in accordance with the following publications: (a) Student-Faculty Handbook 1992-1993, pp44-46, by the Graduate School of Biological Sciences of the Mount Sinai School of Medicine; (b) A memorandum regarding the *Instructions for Preparing the Ph.D. Dissertation*, by Robert Goldstein from the Office of the Registrar, the Graduate School and University Center of the City University of New York.

Chapter one provides a general introduction of tumor rejection antigens, and a brief history of how gp96 was discovered. The rationale for this work is presented.

Chapters two and three contain the results and are written as independent papers under the close supervision of Prof. Pramod K Srivastava. Chapter two describes the ATPase activity and peptide binding property of gp96, and highlights the proposal that gp96 facilitates the assembly of MHC class I-peptide complex. This chapter is due to appear in the EMBO Journal. Permission from the publisher (Oxford University Press) was granted.

Chapter three describes the association of gp96 with MHC class I and has been submitted to Science (Washington D.C.) for publication.

Chapter four discusses our results with gp96 in light of our knowledge of MHC class I-peptide assembly. The possible structural basis of tumor-specific immunogenicity of gp96 is discussed.

The appendix consists a brief review on antigen presentation by MHC class I. A unified list of references is provided at the end.

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ABBREVIATIONS

ABC	ATP-binding cassette
β2m	β2 microglobulin
CTL	cytotoxic T lymphocyte
ER	endoplasmic reticulum
gp96	96-kilodalton glycoprotein
GRP	glucose regulated protein
HPLC	high pressure liquid chromatography
HSP	heat shock protein
kDa	kilodalton
MCA	methylcholanthrene
MHC	major histocompatibility complex (gene)
	major histocompatibility component (protein)
TAP	transporter-associated protein
TRA	tumor rejection antigen
SDS-PAGE	sodium dodecylsulfate polyacrylamide gel electrophoresis
TLC	thin-layer chromatography

CHAPTER 1. INTRODUCTION

Section #1.1 Tumor-specific antigens

The enthusiasm for searching for tumor-specific antigens has been due largely to the expectations that tumors, like infectious diseases, can be prevented and perhaps cured immunologically. The existence of such antigens was first demonstrated by the observation that inbred mice vaccinated with an autologous or syngeneic tumor became immunologically resistant to live challenge with the same tumor (Gross 1943; Foley 1953; Baldwin, 1955; Prehn and Main 1957; Klein et al, 1960; Old et al. 1962; also see Srivastava and Old, 1988). Although the observations were originally made with tumors induced by carcinogens, they have since been extended to a broad spectrum of spontaneously arising tumors as well (Baldwin, 1966; Vaage, 1968; Morton et al, 1969; Carswell et al, 1970; Klein and Klein, 1977; Middle and Embleton, 1981). The demonstration of tumor-specific antigens contributed to the immune surveillance theory proposed by Burnet 20 years ago (Burnet, 1970, 1971; also see Ehrlich, 1909; Burnet, 1957). According to this theory, one of the major functions of T lymphocyte-mediated immunity is to eliminate tumor cells as they arise and this occurs because tumor cells express new epitopes whereby they are recognized as foreign. Experiments in the last two decades have modified the original version of tumor surveillance theory (Stutman, 1979; Rygaard and Povlsen, 1976) and as a consequence, immunosurveillance may no longer be considered to be confined to specific immune response, but also may include non-specific effectors.

Two recent discoveries have reinforced our notion of tumor-specific antigens. One is the clear-cut demonstration that common human cancers usually harbor tumor-specific mutations in predictable sets of genes such as oncogenes and tumor suppresser genes (see Urban and Schreiber, 1992) as well as in unpredictable sites (see Boon, 1992). The second is the discovery that any intracellular mutant molecules could be potentially presented to T cells and thus act as antigens (see Townsend and Bodmer, 1989).

At this point, it is important to make a clear distinction between tumor specific molecules and tumor specific antigens. The former simply means any molecules with tumor specific alterations occurring during the course of transformation. This encompasses a large number of molecules involved in cellular proliferation and differentiation notably proto-oncogenes and tumor suppresser genes. Mutations of such genes contribute to malignancy, and the mutant forms are often not found in normal cells and thus are tumor-specific molecules. The concept of tumor specific antigens however emphasizes the point that those molecules not only have to be "tumor-specific", *but also be capable of eliciting immune response.* The outcome of such immune response is expected to be beneficial and result in tumor regression.

The pace of identification of tumor specific molecules is much faster than the pace of demonstration of whether those molecules are actually immunogenic. For example, mutations of *ras* oncogene and p53 tumor suppresser genes are heavily represented in human malignancy (Levine et al, 1991; Wogan, 1992), but the ability of host to mount immune response against their products is just beginning to be examined. This is why there is a long list of tumor-specific molecules but

the real tumor antigens, although having been vigorously sought, remain only a few. This does not mean however that most of tumor specific molecules are not immunogenic.

Immune response to cancer can be monitored by antibody reactivity, T cell reactivity or in case of experimentally induced cancers, by tumor rejection assays (see Srivastava, 1991). Although a small number of tumor-specific molecules, which elicit an anti-tumor antibody response have been identified (Table 1.1), the role of anti-tumor antibody response in tumor immunity is largely unclear. Surprisingly, antibodies to some molecules, which are not tumor-specific, have been shown to be significantly therapeutic in case of some tumors (for a review, see Houghton et al, 1991). T cell response has been shown to be the major immunoprotective response to cancer and this response has been monitored largely by the presence of anti-tumor cytotoxic T lymphocytes (CTL). However, identification of CTL-recognized tumor antigens has been difficult because of a lack of a general method for identification of T cell recognized antigens. Only recently has progress been made in this direction (See review by Boon, 1992). Table 1.2 shows a list of the tumor antigens known to be recognized by anti-tumor CTLs.

Tumor rejection assays, in which animals are immunized by a given antigen and then challenged with tumor cells, in order to identify a tumor protective antigen, have also been used for identification of immunogenic tumor antigens. Table 1.3 lists the antigens identified in this manner. For obvious reasons, this approach has been attempted only in cancers of experimental animals, although human counterparts have been identified in some instances. The work described in this

dissertation addresses one of the tumor specific antigens identified by the tumor rejection assay.

Table 1.1 Non-viral, structurally characterized antigens of solid tumors identified by antibody reactivity

Antigen	Source	Relationship to known proteins	Reference
gp96	rat hepatoma	HSP	Srivastava and Das, 1984
gp95/p97	human melanoma	Melanotrans-ferrin	Woodbury et al, 1980 Real et al, 1984; 1988 Houghton et al, 1991
GM1, GM2 GD2, GD3	"	Gangliosides	Livingston et al, 1987 Furukawa & Lloyd, 1990 Houghton et al, 1991
p53	human breast cancer	P53 tumor suppresser gene	Davidoff et al, 1992
p53	Meth A sarcoma	"	DeLeo et al, 1979 Jay et al, 1979
gp75	"	Tyrosinase	Vijayasaradhi and Houghton, 1991 Vijayasaradhi et al, 1990

Table 1.2 Antigens recognized by anti-tumor CTLs

Antigen	Source	Structure or tissue distribution	References
P91A	p815 mastocytoma	60 kDa point mutation (R to H)	Lurquin et al, 1989
P35B	"	point mutation (S to N)	Szikora et al, 1990
P198	"	23.5 kDa point mutations (A to T)	Sibille et al, 1990
P1.A	"	no mutations	Van den Eynde et al, 1991
Mucin		pancreatic tumor	Barnd et al, 1989
MAGE1	human melanoma	tissue-specific expression	Van der Bruggen et al, 1991
MAGE2	"	"	Coulie et al, 1993
MAGE3	"	"	
Tyro-sinase	human melanoma	tissue-specific expression	" "

Table 1.3. Molecules that elicit protective immune response to cancer

Protein	Source	References
p90	SV40 transformed cells	Anderson et al 1977
gp70	MCA sarcoma	Zbar et al 1981
gp100/gp96	Rat hepatoma	Srivastava & Das,1984
gp96	MCA sarcomas	Srivastava et al, 1986
p84/86	MCA sarcomas	Ullrich et al, 1986
p67	*ras* transformed fibroblasts	Konno et al, 1989
hsp70	MCA sarcoma	Udono and Srivastava, 1993b
B700	B16 melanoma	Hearing et al, 1986
p67	AK5 histiocytoma	Khar et al, 1992
gp76	UV sarcoma	Ransom et al, 1981
gp30	colon sarcoma	Sato et al, 1987
p75/82	MCA sarcomas	DuBois et al, 1982
gp175	MuLV leukemia	Rogers et al, 1984
p50	*ras* transformed cells	Torigoe et al, 1991

Section #1.2 Identification of gp96 heat shock proteins as tumor rejection antigens

By using a panel of BALB/c fibrosarcomas (Meth A, CMS-5, etc.) induced by methylcholanthrene (MCA) and the classical tumor transplantation rejection assay in syngeneic inbred mice, Srivastava and colleagues (Srivastava and Das, 1984; Srivastava et al, 1986; 1987; Palladino et al, 1987) isolated a tumor specific rejection antigen (TRA). This antigen is a glycoprotein with molecular weight of approximately 96 kilodaltons (kDa), named gp96. Injection of gp96 purified from Meth A tumor cells rendered mice immune to subsequent Meth A challenge. Normal cells as well as other tumors examined so far express gp96 protein. However, injections of mice with gp96 purified from normal tissues are not protective against tumors tested (Udono and Srivastava, 1993a), and mice immunized with Meth A gp96 are only protected against Meth A challenges not against CMS-5, an antigenically distinct line of MCA induced BALB/c fibrosarcoma. Conversely, gp96 purified from CMS-5 can only immunize mice against CMS-5 and not against Meth A.

The above data were originally interpreted to mean that tumor cells harbor specific mutations in gp96 genes and the pattern of mutations is different from tumor to tumor (Srivastava et al, 1987; Srivastava and Old, 1988).

In order to provide a structural definition for these speculations, extensive cloning and sequencing were carried out. Two surprising observations resulted (for reviews, see Srivastava and Maki, 1991; Srivastava and Heike, 1991). First, no tumor specific alterations of gp96

including gp96 primary cDNA sequence, expression level, and subcellular localization were detected (Srivastava et al, 1987 and unpublished data). Secondly, gp96 was observed to be closely related or even identical to a constitutively expressed and stress-inducible ER lumen protein, ERp99 or grp94. Several line of evidence have shown clearly that gp96 is a heat shock protein. The expression of gp96 can be upregulated by exposing cells with stress such as depleting glucose in the culture media, treatment of cells with 2-mercaptoethanol, introducing misfolded proteins in the ER and heat shock (Maki, 1991; Lee et al, 1984; Kozutsumi, 1988). Moreover, the promoter regions of human gp96 genes harbor heat shock responsive elements, a hallmark of HSPs (Maki et al, 1990). The observation that gp96 is both a TRA and a HSP was puzzling because HSPs are among the least evolutionarily diverse proteins present across vertebrates (Lee et al, 1984; Mazzarella and Green, 1987; Maki et al, 1990) and are not expected to confer to individually distinct tumor-specific immunities.

Interestingly, since the original observation was made with gp96, a number of other TRAs, which are immunogenic against tumor challenges have been identified and a significant proportion of these TRAs have turned out to be HSPs (see Table 1.4). Of particular note, in none of these cases have tumor-specific genetic alterations been identified.

Table 1.4. Molecules that elicit protective immune response to cancer and their relationship to other proteins

		Relationship to known proteins			
Protein	Source	Viral proteins	HSPs	Others	References
P90	SV40 transformed cells	T antigen			Anderson et al 1977
gp70	MCA sarcoma	MuLV gp70			Zbar et al 1981
gp100	Rat hepatoma		gp96		Srivastava and Das, 1984
gp96	MCA sarcomas		gp96		Srivastava et al 1986
p84/86	MCA sarcomas		hsp90		Ullrich et al, 1986
p67	*ras* transformed. fibroblasts[a]		hsp70		Konno et al, 1989
hsp70	MCA sarcoma		hsp70		Udono and Srivastava, 1993b
B700	B16 melanoma		albumin-like[b]		Hearing et al, 1986
p67	AK5 histiocytoma		albumin like[b]		Khar et al, 1992
gp76	UV sarcoma			?	Ransom et al, 1981
gp30	colon sarcoma			?	Sato et al, 1987
p75/82	MCA sarcomas			?	DuBois et al, 1982
gp175	MuLV leukemia			?	Rogers et al, 1984
p50	*ras* transformed cells			?	Torigoe et al, 1991

[a]Role of HSPs is strongly suggested, not formally proven. [b]Albumin is heat inducible in fetal liver though not in adult tissue (Srinivas et al, 1987).

The observation of specific immunogenicity of cognate gp96 preparations in light of lack of a corresponding structural difference in gp96 genes suggest that either non-protein components (e.g. carbohydrates) of gp96 or some unidentified components, other than gp96, which are present in gp96 preparations, were responsible for immunogenicity. The role of N-linked glycosylation in immunogenicity was ruled out by the demonstration that enzymatically deglycosylated preparations of gp96 was immunogenic in TRA assays (Srivastava et al, unpublished). The possibility that protein components other than gp96 may be responsible was addressed. The homogeneity of gp96 preparation was tested and it was shown to be homogenous by the following criteria.

(i) Silver-staining of SDS-PAGE of gp96 preparations showed a single band. In preparations where more than one band were detected, each band was recognized by monoclonal and polyclonal antibodies against gp96.

(ii) Edman degradation of gp96 preparation detects a single amino-terminus. The strength of the signal was found to be corresponding to the amount of protein applied.

(iii) Application of immunogenic gp96 preparation from Meth A sarcomas over a gp96 antibody immunoaffinity column, resulted in loss of the immunogenic activity in the unbound fraction.

Collectively, the observations indicate that the immunogenic moiety was not a mere contamination of gp96 preparation. It was either an unidentified structural component of gp96, or it was a low molecular weight molecule tightly associated with gp96.

At this time, it was demonstrated that immunity elicited by gp96 was $CD8^+$ T lymphocyte-mediated (Srivastava et al, 1990; Udono and Srivastava, 1993c). It is established that $CD8^+$ T lymphocytes recognize antigenic peptides in the context of MHC class I molecules (Zinkernagel and Doherty, 1974; see also Yewdell and Bennink, 1992). Our attention was therefore drawn to the possibility that gp96 may be acting as a carrier of antigenic peptides. Such possibility is consistent with the observation that gp96 is a HSP and HSPs are known for the abilities to bind (chaperone) a diverse array of molecules (see review by Ang et al, 1991, Gething and Sambrook, 1992).

Section #1.3 Gp96: from a tumor rejection antigen to a protein chaperone?

In light of the preceding discussion, a hypothesis has been proposed to explain the paradox that gp96 molecules with identical sequences can elicit specific immunity against different tumors (Srivastava and Maki, 1991; Srivastava and Heike, 1991). It is suggested that (i) gp96 is ***not an antigenic molecule per se but acts as a carrier*** of antigenic peptides. (ii) Because gp96 binds to peptides and is localized in the lumen of the ER, the physiological function of gp96 is to promote the assembly of peptides with MHC class I-β2 microglobulin complex (Fig. 1.1).

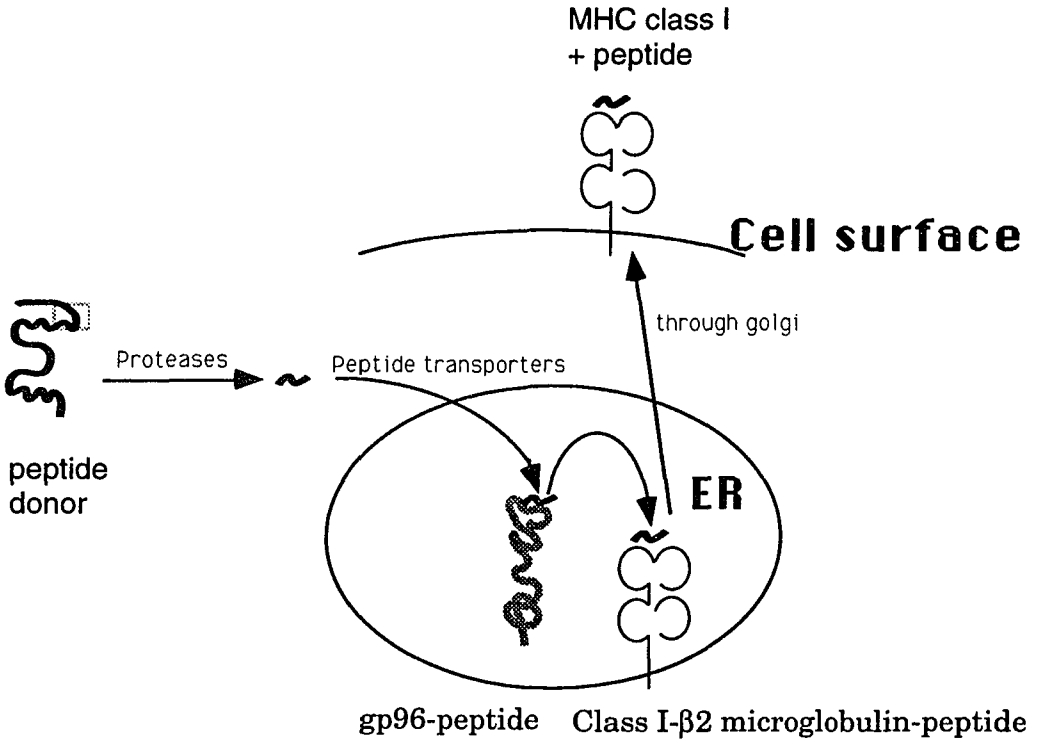

Figure 1.1 A hypothetical view on the role of gp96 in antigen presentation by MHC class I. Peptides generated in the cytosol are transported into the lumen of the ER by transporter-associated proteins (TAP) (Monaco, 1992). Gp96 then concentrates or protects peptides by virtue of its ability to bind to them. Gp96 facilitates the assembly process by transferring its associated peptides to MHC class I-β2 microglobulin complex.

Section #1.4 Context of present work

When I started my work two and half years ago, the intellectual atmosphere was just about ripe to test some central elements of the hypothesis that gp96 is associated with peptides and is involved in MHC class I-peptide assembly. It became clear that the assembly of MHC class I-peptide complex occurs in the lumen of the ER; peptides are important for the folding and transport of MHC class I heavy chain; and a number of laboratories were speculating about the presence of ATP-dependent protein chaperone(s) for MHC class I *in vivo*.

In this context, I proceeded to address the following questions:

(i) Are there any endogenous peptides associated with gp96?
(ii) Does gp96 bind ATP?
(iii) Is gp96 an ATPase?
(iv) Does gp96 associate with MHC Class I?

Answers to these questions are not only critical to explain the structural basis of the tumor-specific immunogenicity of gp96, but also important to understand the mechanism of MHC class I-peptide assembly.

CHAPTER 2. Tumor Rejection Antigen Gp96/Grp94 Is An ATPase: Implications for protein folding and antigen presentation*

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(*The EMBO Journal, 1993, 12, in press;

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Immunization of mice with gp96 / grp94 heat shock proteins (HSPs) elicits tumor - specific cellular immunity to the tumors from which gp96 is isolated. However, the cDNA sequence of gp96 is identical among tumors and normal tissues. This raises the question regarding the structural basis of the specific immunogenicity of gp96. As HSPs bind a wide array of molecules including peptides, we have proposed that gp96 may not be immunogenic *per se*, but may chaperone antigenic peptides. Further, gp96 is localized predominantly in the lumen of the endoplasmic reticulum (ER) suggesting that it may act as a peptide - acceptor and as accessory to peptide-loading of MHC class I molecules. We demonstrate here that gp96 molecules contain ATP - binding cassettes, bind ATP, and possess a Mg^{2+} dependent ATPase activity. Gp96 preparations are also observed to contain tightly - bound peptides, which can be eluted by acid extraction. These properties of gp96 are consistent with its proposed roles in chaperoning antigenic peptides and in facilitating MHC class I - peptide assembly in the ER lumen. We

present a model to explain how interaction of gp96 with MHC class I may result in transfer of peptides to the latter.

Introduction

Immunogenicity of tumors was first demonstrated convincingly in chemically - induced sarcomas of inbred mice (Gross 1943, Foley 1953, Prehn and Main 1957, Klein et al. 1960, Old et al. 1962). A search for the molecules which mediate this individually - distinct immunogenicity led to identification of gp96 molecules as tumor rejection antigens (TRAs) in methylcholanthrene - induced sarcomas of inbred BALB/c mice (Srivastava et al. 1986, Palladino et al. 1987, Feldweg and Srivastava 1993). Mice immunized with 5-10 μg of purified gp96 became immune to subsequent challenges with the tumor from which gp96 was isolated, but not to challenges with antigenically distinct tumors. Immunodepletion of a gp96 preparation with an anti-gp96 antibody to gp96 depletes it of antigenicity (Srivastava et al. 1986). Rabbit antisera revealed the presence of gp96 in normal tissues as well (Srivastava et al. 1986). However, gp96 isolated from normal tissues does not elicit tumor immunity (Udono and Srivastava 1993). These observations suggested that there might be mutations in gp96 genes of tumors and that these mutations differ from one tumor to another. However, sequencing of gp96 cDNAs from BALB/c spleen, and fibrosarcomas Meth A and CMS5 did not reveal any tumor-specific, individually distinct mutations (see Srivastava and Maki 1991). These observations raise a question as to the structural basis of specific immunogenicity of gp96.

Comparison of the gp96 sequence to known sequences revealed significant homology with the heat shock protein (HSP) hsp90 and possible identity with the glucose - regulated protein grp94 (Srivastava et al. 1987, Mazzarella and Green 1987, Lee et al, 1984, Maki et al. 1990, 1993). Hsp90 has been reported to bind a diverse range of cellular proteins and a protein chaperoning role has been attributed to it (Shaknovich et al, 1992, Wiech et al, 1992). A role for gp96 / grp94 in protein folding has also been suggested on basis of the observation that it is induced by accumulation of misfolded proteins in the endoplasmic reticulum (ER) (Kozutsumi et al, 1988). In lack of sequence differences in gp96 genes between tumors and normal tissues in spite of the tumor - specific antigenicity of gp96, we proposed (Srivastava and Maki 1991, Srivastava and Heike 1991) that gp96 may not be immunogenic per se, but may be a carrier of antigenic peptides. In view of the predominant localization of gp96 in the ER (Booth and Koch 1989), and our observation that the immunity elicited by gp96 is mediated through $CD8^+$ T lymphocytes (Udono and Srivastava 1993), we proposed that gp96 acts as a peptide-acceptor for peptides transported to the ER and enables peptide-loading of MHC class I.

In light of the fact that peptide - charging of MHC class I is an ATP - dependent process (Levy et al. 1991, Luescher et al. 1992), we have examined the ability of gp96 to bind peptides and ATP. We demonstrate that peptides are indeed associated with gp96 and that gp96 binds ATP and is an ATPase. These observations make gp96 a logical candidate for facilitating peptide - charging of MHC class I and provide a structural basis for the tumor - specific immunogenicity of gp96.

Results

Gp96 is an ATP - binding protein

The deduced amino acid sequence of the murine gp96 was screened for the presence of motifs which are associated with ATP - binding. The ATP - binding consensus sequences , type A and type B, as proposed by Walker et al. (1982) and refined by Chin et al. (1988) were used. One type A (aa 217 - 224) and two down stream type B sequences (aa 231 - 241 and 303 - 313) were identified (Fig. 2.1). Within the A type sequence, there are three amino acid residues between Gly-217 and Gly-221 instead of four as proposed in the A type consensus sequence. The two B type sequences have one mismatch each, in the hydrophobic regions. Flaherty et al. (1991) have suggested an additional sequence (ILV)X(ILVC)DXG(TSG)(TSG)XX(RKC) as a fingerprint characteristic for nucleotide - binding proteins, based on the crystal structure of the heat shock protein hsc70. This motif is conserved in hsc70, grp78 and dnaK from *E. coli*, but is not found in the ATP - binding protein groEL. This sequence was not detected in gp96.

ATP-binding by gp96 was measured experimentally by photoaffinity labeling of purified gp96 by 8-azido γ-^{32}P ATP (Fig. 2.2, lane 1). In parallel, ATP - binding of hsp90 (as a positive control, lane 2) and an un-related 96 kDa protein (as a negative control, lane 3) was also tested. Gp96 was observed to bind ATP (Lane 1) and this binding was inhibited by inclusion of cold 10 mM 8-azido ATP (lane 4), ATP (lane 5), but not cyclic AMP (lane 6), GTP (lane 7) or CTP (lane 8).

		Type A							Type B					
Consensus		(G/A)X4(G/A)(H/K/R)X0-1(T/S/K/R/H)							(H/K/R)X5-8ΦXΦ2(D/E)					
GP96:	217-224	G	-NTL	GR	G	T	231-241	K	EEASD	Y	L	eL	D
							303-313	K	KVEKT	V	W	dW	E
Adenylate Kinase:	15-23	G	GPGS	GK	G	T	108-119	K	IGQPTL	L	L	YV	D
RING 4:	C-terminal	G	PNGS	GK	S	T	C-terminal	K	PCVLILDD	a	S	tL	D
Protease La:	355-362	G	PPGV	GK		T	411-422	K	VGVKNP	L	F	LL	D
GroEL:	164-172	A	MDKV	GK	E	g	241-251	K	AGKD	L	L	IA	E

Figure 2.1. Sequence homology among the putative ATP binding domains of gp96 and other ATP-binding proteins. The consensus ATP binding sites proposed by Walker et al (1982) and modified by Chin et al (1988) are presented. The putative ATP binding sequences of gp96 are aligned with sequences of four other ATP binding proteins : rabbit muscle adenylate kinase (Kuby et al, 1984), human transporter-associated protein 1 (Ring 4) (Trowsdale et al, 1990), *Escherichia coli* chaperonin GroEL (Hemmingsen et al, 1988), and protease La (Chin et al, 1988). Single amino acid codes are used here. F stands for a hydrophobic amino acid (I, V, L, M, Y, W, F); X indicates any amino acids. Lowercase letters represents nonhomologous residues. "-" indicates a gap. The highly conserved residues are boxed.

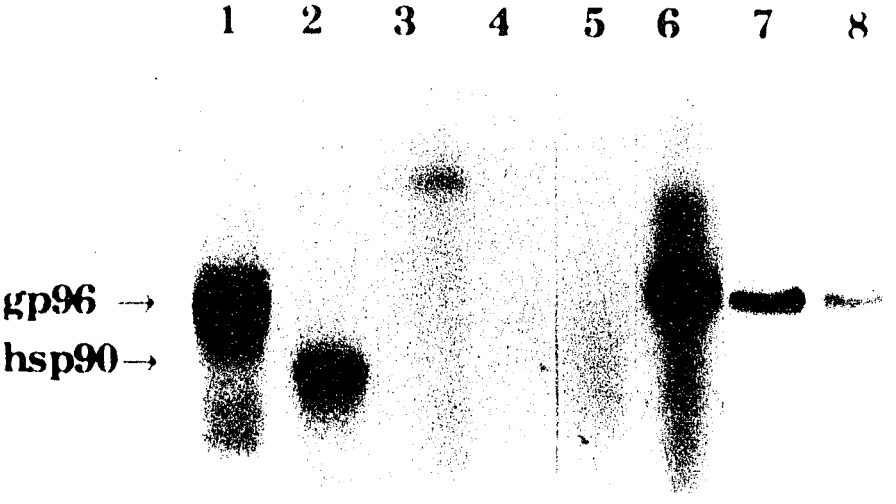

Figure. 2.2. Gp96 binds ATP in vitro. Binding of gp96 (Lane 1), hsp90 (lane 2), or p96 (a Meth A derived protein unrelated to gp96, lane 3) to 8-azido γ-p^{32} was carried out as described in Methods. Lanes 4 through 8 : binding was carried out in presence of 10 mM unlabelled 8-azido ATP (lane 4), ATP (lane 5), cAMP (lane 6), GTP (lane 7) and CTP (lane 8).

To determine if gp96 interacts with ATP *in vivo*, EL-4 cells were labeled with [p^{32}]-phosphate, and gp96 was immunoprecipitated from whole cell lysates of such cells. A phosphate - labeled gp96 band was observed (Fig. 2.3A). These experimental conditions should detect only phosphorylated or ADP-ribosylated proteins or proteins with tightly bound nucleotides. In fact, two other phosphoproteins MHC class I and hsp70 were also precipitated from these extracts with the respective antibodies and were detected by autoradiography. In case of hsp70, a number of bands other than the 70 kDa band are detected in the immunoprecipitate; these proteins are presumably associated with hsp70 because the anti-hsp70 antibody used is quite specific and detects only hsp70 on Western blots.

To determine, if the ^{32}P label in gp96 derives from associated ATP, or if it is a result of a post - translational modification, radioactive adenosine nucleotides were specifically eluted from the immunoprecipitates by large excess of cold ATP, ADP and AMP. It was observed (Fig. 2.3B) that gp96 and hsp70 but not MHC class I contain tightly - bound ATP, ADP and AMP. ADP is the major component eluted from gp96 and hsp70; ATP contributes to only a minor fraction of the total eluted nucleotides. This is consistent with the observations made with Dnak, and the crystallographic analysis of hsp70 (Flaherty et al. 1990). ATP is not detected in abundance presumably because of its rapid hydrolysis.

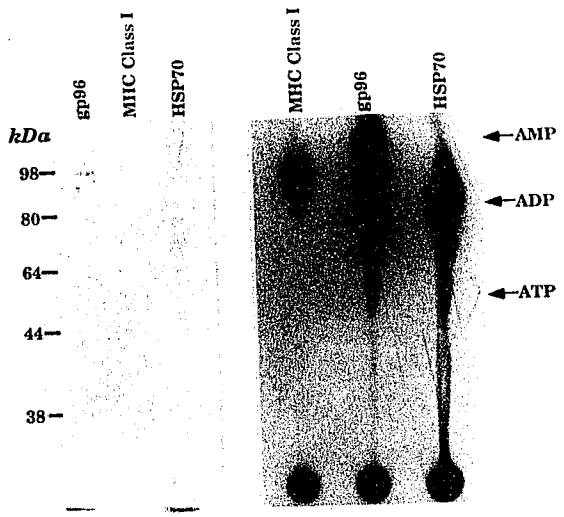

Figure 2.3.

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Figure 2.3. Interaction of gp96 with adenosine nucleotide *in vivo*. **(a)** Immunoprecipitation of phosphate labeled gp96 from ^{32}P-orthophosphate-labeled EL-4 cells. Immunoprecipitation was done with a polyclonal rabbit serum against MHC class I whole molecule (K270, obtained from Dr Per Petersen), rat anti-gp96 monoclonal antibody (clone 9G10) and rat anti-hsp70 monoclonal antibody (clone 7.10) respectively followed by secondary rabbit anti-rat IgG and protein A-Sepharose. **(b)** PEI-thin layer chromatography of gp96-associated nucleotides. Immunoprecipitates were washed and associated nucleotides were eluted and separated by PEI-TLC plates (see Materials and Methods). One µl of 100 µM ATP, ADP and AMP was spotted and localized with the aid of short wave UV lamp and the positions are indicated by arrows. The spot seen in the MHC class I lane on the TLC represents less than 5% of the counts seen in the gp96 or hsp70 lanes. The films were exposed to a non-linear degree in order to show the ATP spots clearly.

Gp96 is an ATPase

Figure 2.4A shows the chromatographic profile of the last step of purification of Meth A gp96 on a Mono Q FPLC column. Gp96 elutes within a broad range of salt concentration (0.45 M-0.6 M NaCl), instead of a sharp peak. However, the protein material in each peak is comprised of apparently homogeneous gp96 as judged by silver - stained gels of these fractions (Figure 2.4B). The fractions were individually assayed for ATPase activity as described in Methods. ATPase activity was clearly detected in the gp96 fractions (Fig. 2.4C). The activity in this experiment is not linear with the concentration of gp96 because this protocol measures net activity rather than the initial velocity of reaction. For subsequent characterization of the ATPase activity of gp96, fraction 6 was used.

To confirm that the ATPase activity was derived from gp96 molecule itself, fraction 6 (from the experiment in Fig. 2.4A) was applied sequentially a number of times, to an anti-gp96 monoclonal antibody column and depleted of gp96. The initial and depleted fractions were characterized by SDS-PAGE and assayed for ATPase activity. It was observed that the loss of ATPase activity was concomitant with loss of gp96 (Fig. 2.5 A, B and C). Application of fraction 6 through an unrelated immunoaffinity column did not deplete the preparation of gp96, nor of ATPase activity (data not shown). These experiments show that the ATPase activity of gp96 resides in the gp96 itself and is not a contaminant.

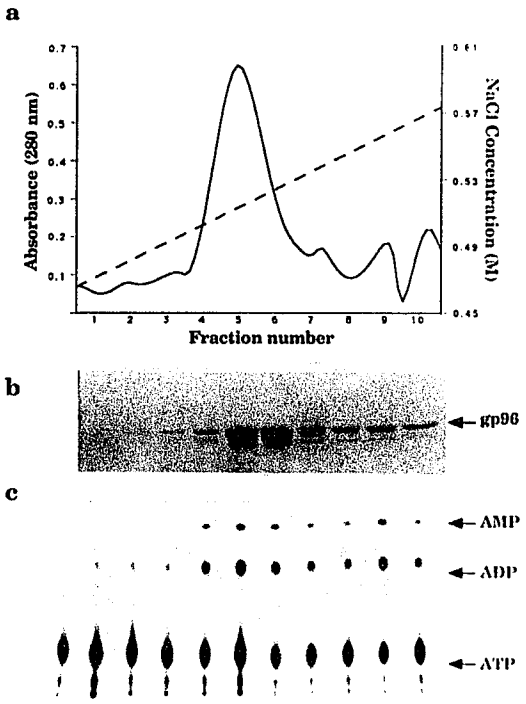

Figure 2.4.

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Figure 2.4. ATPase activity copurifies with gp96. (a) Chromatographic profile of gp96 on Mono Q-FPLC. Proteins bound to Concavalian A-Sepharose column were dialyzed against 5 mM sodium phosphate buffer, pH 7.0 and applied to Mono Q-FPLC column. Bound proteins were eluted with a linear 0-1 M NaCl gradient. Gp96 elutes at NaCl concentration between 0.45 M and 0.6 M. **(b)** Silver-stained SDS-polyacrylamide gel (PAGE) of MonoQ-FPLC purified fractions shown in (a). Fractions were applied to 10% SDS-PAGE and silver-stained. Lane 1 to 10 represent 3 µl aliquot of each 1 ml fraction. **(c)** *In vitro* ATPase activity of purified gp96. 10 µl of gradient fractions shown in (a) were assayed for ATPase activity using 2 µM α-^{32}P-ATP (see Materials and Methods). Reaction products were analyzed by polyethyleneimin (PEI) thin-layer chromatography and autoradiography. The positions of ATP, ADP and AMP are shown.

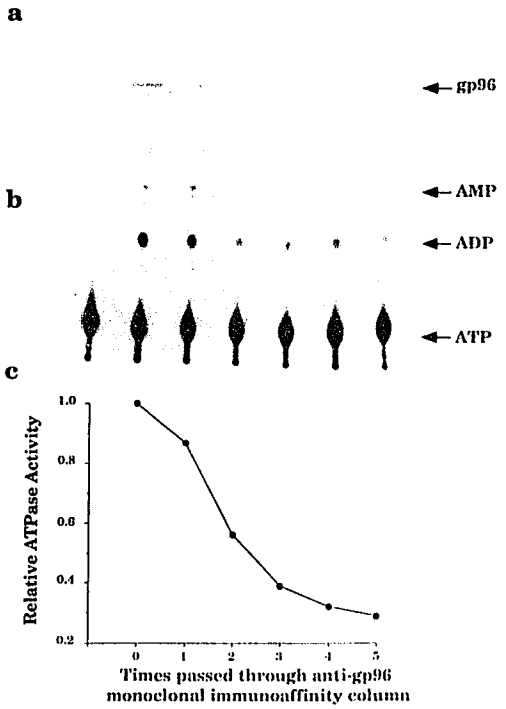

Figure. 2.5. ATPase activity resides in the gp96 molecule itself. **(a)** Depletion of gp96 using immunoaffinity column. Monoclonal antibody was coupled to Protein G-Sepharose column. Same volume of original materials (~ 0.5 μg) and materials coming from the column each time were applied to 10% SDS-PAGE and silver-stained. **(b)** Samples before (0.5 μg) and after depletion were assayed for ATPase activity by incubating with 20 μM α-^{32}P-ATP for 30 minutes. **(c)** Quantitative analysis of results in (b). Thirty pmol ATP was hydrolyzed by 0.5 μg gp96 before the immunodepletion, and the relative ATPase activity was defined as 1.

Effect of temperature, pH and divalent cations on the ATPase activity of gp96

ATPase activity of gp96 was assayed at different temperatures at a pH of 7.2 and was found to be optimal at 42°C (Fig. 2.6A). A temperature optimum higher than 37°C has been noted for some other HSPs as well; for example, dnaK, a bacterial hsp70, shows optimal ATPase activity at 50-55°C (Liberek et al, 1991). Further, the ATPase activity of gp96 was observed to be optimal at acidic pH (Fig. 2.6B). The divalent cation dependence of gp96 ATPase was tested and the activity was found to be dependent on exogenous Mg^{2+} but not on Ca^{2+} (data not shown). This was confirmed by the use of divalent cation chelators EDTA and EGTA in the reaction mixture. EDTA, which is a general divalent cation chelator had a significantly stronger inhibitory effect on the ATPase activity of gp96 than EGTA, which is a Ca^{2+} specific chelator (Fig. 2.6C). The other ER luminal HSP, the grp78 is similar to gp96 in its requirement for divalent cations (Kassenbrock and Kelly, 1989): grp78 requires Mg^{2+}, but not Ca^{2+} for its ATPase activity and indeed, the activity is inhibited by the presence of Ca^{2+}.

A substrate saturation experiment was done to determine the kinetics of ATPase activity of gp96. When the ATPase activity was assayed in the presence of increasing concentrations of ATP, the velocity was found to follow first order kinetics (Fig. 2.7 and inset), suggesting that gp96 has a single ATPase active site. No evidence of co-operativity was observed. Under the conditions of this experiment, the ATPase activity of gp96 has a Km of 8 μM and the turn over rate about 0.08 mole/min/mole. These characteristics suggest that ATP hydrolysis by gp96 plays a regulatory role (see Discussion).

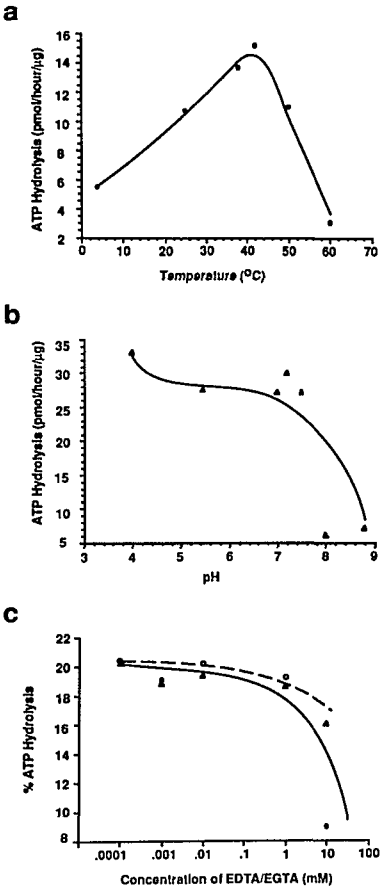

Figure 2.6. Effect of temperature, pH, and divalent cations on the ATPase activity associated with gp96. (a) Effect of temperature on the rate of ATP hydrolysis by gp96. **(b)** Optimization of pH. 570 ng purified gp96 was assayed for ATPase activity in the presence of 20 mM NaCl, 2 mM $MgCl_2$ and 20 mM of various buffers (pH 4.0 Acetate, pH 5.5-6.0 MES, pH 7.0-7.2 HEPES, pH 7.5-8.8 Tris). **(c)** ATPase activity is inhibited by divalent cation chelators. Purified gp96 was assayed for ATPase activity in the presence of 20 mM MES pH 6.0, 20 mM NaCl and increasing concentrations of EDTA (solid line) or EGTA (dotted line).

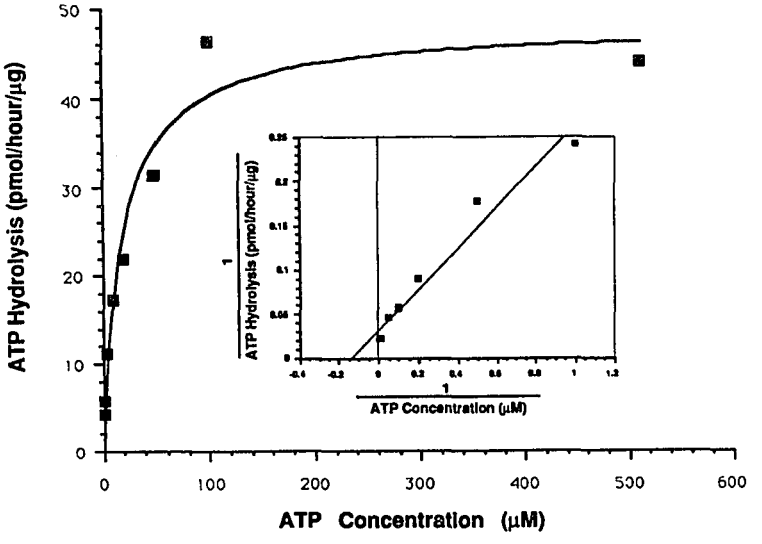

Figure 2.7. Kinetic study of gp96 ATPase activity. Substrate saturation experiment was carried out by incubating purified gp96 (0.862 µg per reaction) with increasing concentrations of α-^{32}P-ATP in 20 mM HEPES buffer pH 7.2 containing 20 mM NaCl and 2 mM $MgCl_2$ at 37°C for 1 hour. Background values in the absence of gp96 have been subtracted. Double-reciprocal plot of the same data is shown in inset. The Km calculated from this experiment is 8 µM.

Protein and peptide modulators of ATPase activity of gp96 and hsp70

Members of the hsp70 family, including grp78 undergo conformational changes and substrate release during ATP hydrolysis (Gething and Sambrook, 1992; Carlino et al, 1992). Moreover, ATP hydrolysis is stimulated by exogenous peptides (Flynn et al, 1989 and 1991). As gp96 and grp78 are the two ER luminal HSPs, we investigated whether ATPase activity of gp96 is also modulated by peptides. Two peptides A and B, which were used for ATPase stimulation assays with grp78 by Flynn et al. (1989) were added in the ATPase assay with gp96. As shown in Figure 2.8A, the ATPase activity of gp96 was not stimulated and was indeed inhibited in the presence of either of the two exogenous peptides. Fifteen additional peptides ranging from 7 to 20 residues in length were also found to have no stimulation effect. A hsp70 preparation isolated from Meth A cells was also tested for ATPase activity and its stimulation by peptides A and B under our experimental conditions. In contrast to the lack of stimulation of gp96 ATPase by peptides, hsp70 ATPase was reproducibly found to be stimulated by approximately 1.5 fold by addition of peptides (Fig. 2.8B). Flynn et al have observed a maximal 4 fold stimulation of the ATPase activity of grp78 by the same peptides under similar conditions. The difference between our results stems perhaps from the fact that we are using a mixed pool of hsp70 molecules rather than a purified grp78 preparation.

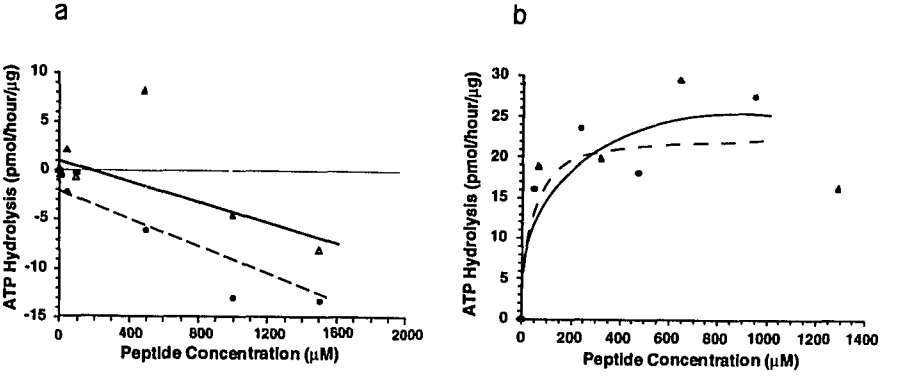

Figure. 2.8.

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Figure. 2.8. Modulation of ATPase activity of gp96 and hsp70 by peptides. (a) Peptides do not stimulate ATPase activity of gp96. 1 μg of purified gp96 was assayed for ATPase activity in the presence of increasing concentration of either peptide A (Δ——Δ) or peptide B (•------•) at 37°C for 1 hour. Both peptides A and peptide B are 15-mers and are derived from vasicular stomatitis virus glycoprotein (peptide A: KRQIYTDLEMNRLGK; peptide B: LSSLFRPKRRPIYKS). Peptide-independent ATP hydrolysis of 50 pmol/hour/μg was subtracted. **(b)** Peptides stimulate ATPase activity of hsp70. Same peptide preparation used in (a) were used. Peptide-independent ATP hydrolysis of 85 pmol/hour/μg has been subtracted.

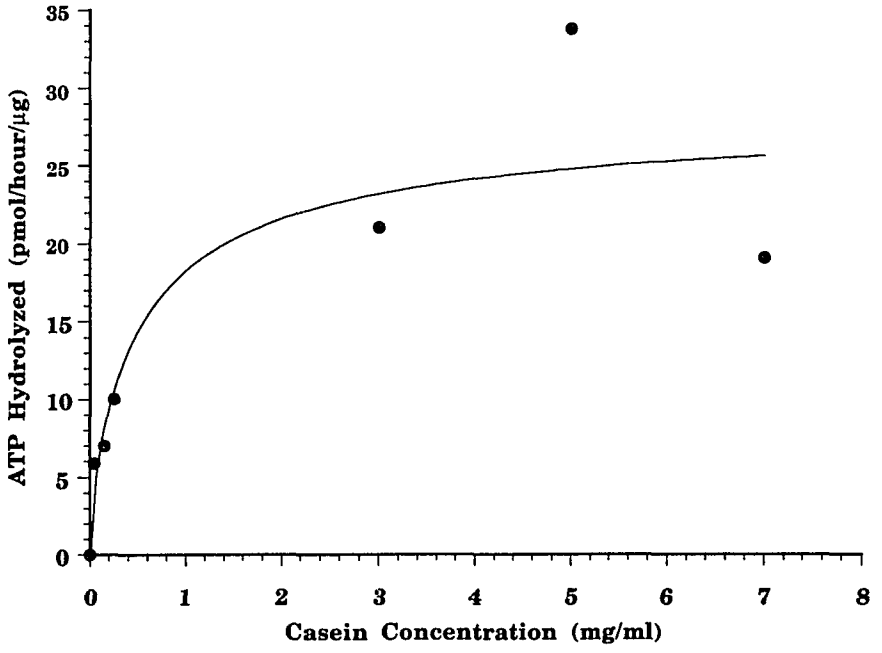

Figure. 2.9. Casein stimulates ATPase activity of gp96. ATPase activity of gp96 was assayed in the presence of increasing concentration of casein. ATPase activity with gp96 alone as well as the minor background ATP hydrolysis associated with casein itself are subtracted from each point.

It has been suggested that the stimulation of ATPase activity of grp78 by peptides reflects the fact that peptides may mimic certain features of the newly synthesized unfolded proteins, whose folding is facilitated by grp78. The stimulation of ATPase activity would thus provide energy for the folding process. However, in contrast to grp78, the bacterial chaperonin groEL (which also hydrolyzes ATP) has been shown to interact only with partially folded proteins or so-called "folding intermediates" or "molten globules" (for reviews, see Gething and Sambrook, 1992; and Ang et al, 1991). Thus, groEL does not recognize either completely folded or totally unfolded polypeptides, nor does it bind peptides. The possibility that the ATPase activity of gp96 is modulated by similar interactions was therefore pursued, and dephosphorylated casein was tested for this purpose. Casein is a soluble protein with a number of exposed hydrophobic surfaces and has been used as a competing substrate for groEL binding (Langer et al, 1992). It was observed that similar to the stimulation of hsp70 ATPase by peptides, casein stimulates the ATPase activity of gp96 by ~2 fold (Fig. 2.9).

Gp96 is associated with peptides

To identify peptides associated with gp96, 10^9 Meth A cells were metabolically labeled with ^{35}S methionine (200 μCi/ml) and gp96 was isolated. The purified preparation was extracted with 10% acetic acid and the low molecular weight material isolated by Centricon centrifugation (Centricon 10, which will allow passage of molecules of 10,000 d or less), as described in Methods. This material was applied to a C_{18} reverse phase HPLC column and eluted on an acetonitrile gradient. Individual fractions were collected and counted. A number of ^{35}S methionine-

labeled distinct peptide peaks were obtained (Fig. 2.10A). However, as this material was insufficient for quantitative analysis, we purified 3 mg gp96 from mammalian liver and acid-extracted it as before. The peptides eluted from it were applied to a C_{18} column. Figure 2.10B shows that a number of peptide peaks were obtained. Some of the peaks have been partially characterized by mass spectroscopy and have been determined to be peptides of heterogeneous size (molecular masses between 400 and 2000 atomic mass units, data not shown). An approximate quantitative analysis of the eluted peptides suggests that gp96 and peptides are present in roughly equimolar stoichiometry, assuming that the average peptide has a molecular mass of 1,000 d. It should be noted that the eluted peptides are not merely loosely associated with gp96, but are tightly bound to it. The purified gp96 preparation was obtained after a number of steps which include elution on a salt gradient on which gp96 elutes at approximately 0.5 M NaCl. The final gp96 preparation may be used immediately for acid - extraction or may be stored in this high salt buffer for several hours in presence of high concentrations of protease inhibitors (2 mM PMSF, 10 μM leupeptin) at 4°C, before acid-extraction. In either case, we routinely centrifuge the purified preparations in the high salt buffer through a Centricon 10 immediately preceding acid-extraction and do not find any peptides in the low molecular weight fraction. Thus, gp96 contains tightly bound peptides, apparently in the same manner and by the same criteria as the MHC class I and class II molecules (Falk et al. 1990, Rotzschke et al. 1990, Rotzschke and Falk 1991).

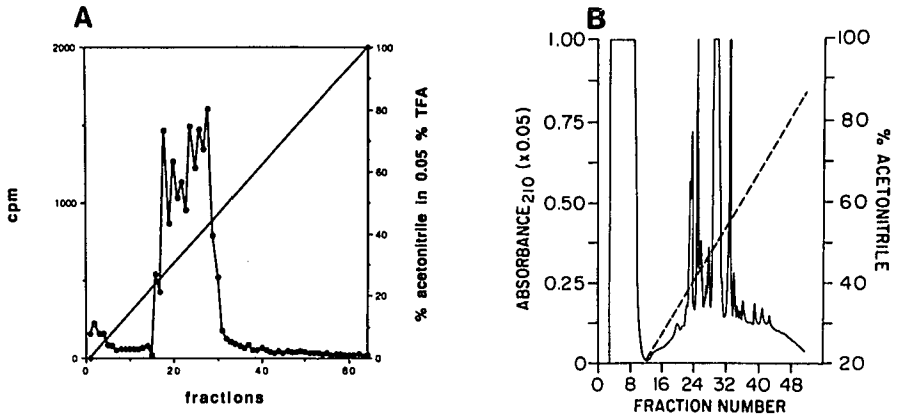

Figure. 2.10. Gp96 is associated with heterogeneous peptides. Purified gp96 was extracted with 10% acetic acid. Peptides eluted from gp96 were separated through Centricon 10 and separated on a C_{18} HPLC column, as described in the Materials and Methods. **(a)** peptides eluted from gp96 derived from $1X10^9$ Meth A cells metabolically labeled with S^{35}-Met. **(b)** Peptides isolated from macaque liver gp96.

Discussion

ATPase activity of gp96 and other HSPs

Gp96/grp94 is the single most abundant component of the lumen of the ER, but no *in vivo* function or enzymatic activity has been assigned to it as yet (Pelham 1986). Our results show novel properties of this molecule and suggest for it a crucial role in antigen presentation by MHC class I molecules and in tumor immunity. We demonstrate that gp96 binds ATP and is a Mg^{2+} dependent ATPase. The deduced amino acid sequence of gp96 contains ATP-binding motifs, and gp96 binds ATP in vitro and in vivo (Fig. 2.2 and 2.3). The Km of the ATPase activity was calculated as 8 μM and the maximum velocity as 50 pmol/hour/μg (Fig. 2.7). This corresponds to a turnover rate of 0.08 mole/min/mole. These characteristics of the gp96 ATPase fall within the range of corresponding parameters for the hsp70 ATPases. For example, the ATPase activity of the E. χολι hsp70 dnaK has a Km value of about 20 μM and a turn over rate of 0.2 mole/min/mole (Liberek et al, 1991). Grp78, an hsp70 member of the ER, has a Km of about 0.1 μM and a turn over rate of approximately 0.34 mole/min/mole (Kassenbrock and Kelly, 1989). A comparison of the turnover rates of ATPase activities of gp96, DnaK and grp78 with the corresponding values for a non-HSP such as adenylate kinase is revealing. The turnover rate of the ATPase activity of rabbit muscle adenylate kinase is 2×10^6 mole/min/mole (calculated from Hampton and Slotin 1975); in comparison, the turnover rate of HSP ATPases, as described above, is several orders of magnitude lower. This indicates that ATP hydrolysis by HSPs has a significantly dissimilar

biological purpose from ATP hydrolysis by non-HSPs such as adenylate kinase and the transporter proteins; specifically, the ATPase activity of HSPs may mediate a regulatory rather than a transporting or modifying function.

A comparison of the ATPase activities of the two HSP-ATPases of the ER lumen, grp78 and gp96 reveals critical differences. The ATPase activity of grp78 is stimulated by peptides, while that of gp96 is inhibited or unaffected. Further, ATPase activity of grp78 is unaffected by the presence of other proteins such as casein, while the ATPase activity of gp96 is stimulated by casein. These differences hint towards different, perhaps complementary roles for the two proteins in the ER lumen. Clairmont et al (1992) have demonstrated that grp78 and gp96 are the two major recipients of the pool of ATP translocated into the lumen of the ER. We suggest that the two molecules use the energy from hydrolysis of ATP in divergent ways, as discussed below.

Role of gp96 in folding / assembly of proteins in the ER

Grp78 is generally thought to catalyze correct folding of newly synthesized proteins in the lumen of the ER. The role of gp96/grp94 in this process has remained largely obscure. Like grp78, grp94 has been shown to be induced by accumulation of malfolded proteins in the ER (Kozutsumi et al. 1991) suggesting that gp96 acts as a chaperone. A number of observations have now begun to show this formally. In our analysis of the assembly of MHC class I with β2 microglobulin and peptides, we have observed association of MHC class I heavy chain with gp96 (Li and Srivastava, in preparation). In earlier studies, Navarro et al. (1991) observed that grp78 and gp96 associate with aberrant forms of

glycoprotein b of the herpes simplex virus 1. Similar observations were reported for newly synthesized immunoglobulin chains by Melnick et al. (1992). Schaiff et al. (1992) showed that MHC class II (HLA - DR) α and β chains associate with gp96 in the ER in the absence of invariant chain. This association does not occur in the presence of invariant chain. The observations reported in this paper, that gp96 molecules bind peptides and ATP, and have a regulatory ATPase activity, provide a missing piece of the puzzle and furnish an enzymatic basis for the emerging evidence for the role of gp96 as a *bona fide* chaperone.

There are two main paradigms known for the mechanism by which HSP-chaperones catalyze protein folding or assembly (Rothman 1989, Gething and Sambrook 1992). In one paradigm, typified by grp78, cycles of binding and release of grp78 with short peptide regions of the substrate protein are propelled by the energy provided by ATP hydrolysis and a polypeptide chain emerges fully folded after having gone through a number of transient associations. In concordance with this paradigm, ATPase activity of grp78 is stimulated by peptides, but not by folded intact proteins (Flynn et al. 1989, 91). In another paradigm, typified by the groE proteins, protein substrates bind to the groEL oligomer at a number of sites and the folding proceeds at different portions of the polypeptide substrate by a progressive, ATP-hydrolysis dependent release of different regions of the substrate from the oligomeric groEL (Gething and Sambrook 1992). Another groE protein, the groES modulates the ATPase activity of groEL. In concordance with this paradigm, the ATPase activity of groEL is stimulated by interaction with some proteins, but is not stimulated by peptides.

The ATPase activity of gp96 appears to be distinct from both paradigms, although it shares selected features with each. Gp96 molecules may exist as dimers or tetramers (Srivastava and Das 1984 and unpublished) and like the groEL and in contrast to grp78, the gp96 ATPase is not stimulated by peptides, but is stimulated by casein - a good model for a partially folded protein. It would appear that the ATPase activity of gp96, like that of dnaK and groEL, is modulated by protein-protein rather than protein-peptide interactions. It is conceivable that the ER lumen contains other proteins, which modulate the ATPase activity of gp96 in the same manner as groES modulates the activity of groEL, or DnaJ and GrpE modulate the activity of dnaK. Gp96 and grp78 may also conceivably modulate and collaborate with each other. Sequential collaboration of the two chaperones dnaK and groEL in the folding process is a precedent for such an effort (Langer et al. 1992).

While gp96 and grp78 may have shared roles in the folding process, the divergent regulation of the two ATPases (Figs. 2.8 and 2.9) may suggest an additional, unique role for gp96. Gp96 is among the most recently evolved HSPs. It is not found in yeast or *Drosophila* and appears to have emerged relatively recently by a duplication of the cytosolic hsp90. The major structural differences between hsp90 and gp96, the amino terminal signal peptide and carboxyl terminal -KDEL sequence, mediate ER targeting and retention. The only additional difference between the two molecules is in the ATPase activity of gp96 : although hsp90 binds ATP, it is not an ATPase. It would appear that gp96 evolved to fulfill an ATPase-dependent function in the ER, that may be unique to higher organisms. In light of the immunological effects of vaccination with gp96 (Srivastava and Das 1984, Srivastava et al. 1986,

Palladino et al. 1987, Feldweg and Srivastava 1993) and our observation that gp96 associates with MHC class I (Li and Srivastava, in preparation), we suggest that one of the specific functions of gp96 involves charging of MHC class I molecules with peptides.

Gp96 - accessory to peptide - loading of MHC class I ?

We demonstrate that gp96 is associated with a wide array of peptides (Figure 2.10). In the context of our observation of co-immunoprecipitation of gp96 and MHC class I from metabolically labeled cells (Li and Srivastava, in preparation) and in light of the ATPase activity of gp96, we propose (Figure 2.11) that (a) gp96 acts as a repository of the peptides transported into the lumen of the ER, (b) interaction of gp96 with MHC class I stimulates its ATPase activity, resulting in hydrolysis of gp96 - bound ATP, (c) the energy released from ATP hydrolysis is used for transfer of peptides from gp96 to MHC class I.

An ER luminal chaperone which will facilitate charging of MHC class I with peptides has been invoked by Rothman, Cresswell, Townsend, Kvist and their colleagues (Rothman 1989, Alexander et al. 1989, 1990, Townsend et al. 1990, Levy et 1991). In view of the ability of gp96 to bind peptides and ATP, its ATPase activity, and its localization in the ER lumen (where it is the major component), it is reasonable to suggest this role for gp96.

An ER chaperonin p88/IP90/calnexin has recently been identified (Degen and Williams 1991, Hochstenbach et al. 1992, Galvin et al. 1992). It is a membrane-bound (as opposed to luminal) protein and is associated with partial but not fully assembled complexes of immunoglobulins, T cell receptors and MHC. Hochstenbach et al. (1992)

suggest that p88 might participate in assembly of multi-subunit complexes. It is possible that p88 is involved in folding of MHC class I heavy chain, but there is no evidence of peptide-binding or ATPase activity of p88 and it does not appear to be an accessory to peptide-loading of MHC class I.

Implications for immune response to cancer

The observations that gp96 is associated with peptides, binds ATP and is an ATPase lends strong support to the suggestion that tumor-specific immunogenicity of gp96 (and perhaps other HSPs) does not derive directly from gp96 but from peptides associated with it. The source of the immunogenic peptides from tumor cells clearly lies in altered (mutated) or mis-expressed cellular proteins and the specificity of immunogenicity may result from randomness of mutations (Szikora et al. 1990, Sibille et al. 1990, Van den Eynde et al. 1991, Boon, 1992). Structural characterization of antigenic peptide(s) eluted from gp96 preparations from antigenically distinct tumors will provide further support for this hypothesis.

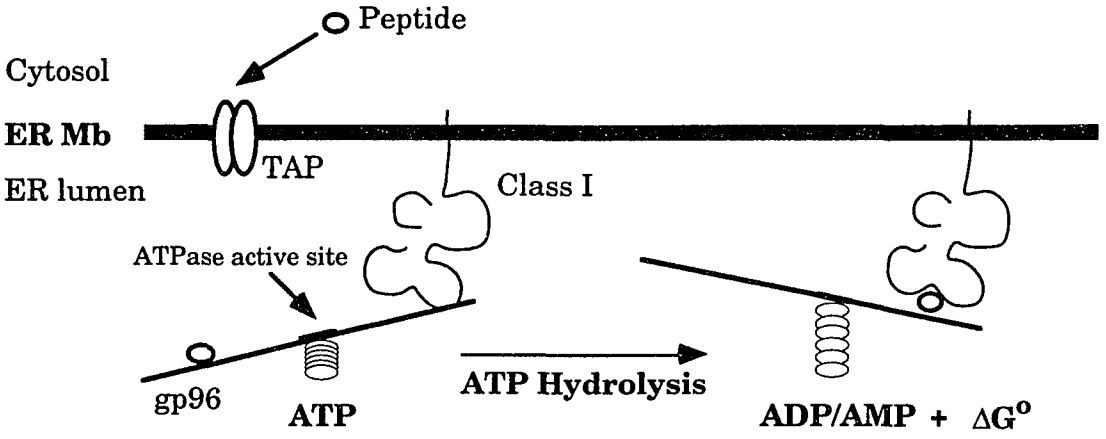

Figure. 2.11. A model for the role of gp96 in MHC class I-peptide assembly. It is postulated that the interaction of MHC class I and gp96 stimulates the ATPase activity of gp96. The free energy (ΔG^o) released due to ATP hydrolysis induce a conformational change in gp96, leading to transfer of peptides from gp96 to MHC class I. TAP denotes transporter - associated proteins.

Materials and methods

Reagents, mice and tumors

All chemicals were purchased from Sigma except where specified. Radiochemicals were from ICN. Cell culture media and reagents were mainly obtained from Gibco-BRL. Monoclonal antibodies against gp96 and hsp70 were purchased from Stressgen. Peptides were synthesized on Applied Biosystems Model 430A peptide synthesizer using FMOC chemistry. Inbred BALB/c mice were obtained from our mouse colonies. EL-4 cells were obtained from ATCC.

Purification of gp96 and hsp70

Gp96 purification has been described by Srivastava et al (1986). Hsp70 was purified essentially as described by Welch and Feramisco (1985).

ATP binding

Purified gp96, hsp90 or other protein (10 μg) in 10 mM Tris pH 7.4, 5 mM $CaCl_2$ and 2 mM 8-azido γ-P^{32} ATP in a final volume of 100 μl is irradiated with a short wavelength (254 nM) UV source from a distance of 8-10 cm for 4 min (Hobson et al. 1984). Samples are microcentrifuged for 5 min to remove large complexes and analyzed by SDS-PAGE.

ATPase assay

ATPase activity was measured by the method of Flynn et al (Flynn et al, 1989 and 1991). Typically, one μg of purified gp96 or hsp70 was incubated with 20 μM [α-p^{32}]-ATP in a reaction volume of 20 μl containing 20 mM HEPES pH 7.2, 20 mM NaCl and 2 mM $MgCl_2$ at 37°C

for 1 hour. One µl of the reaction mixture was then spotted onto polyethyleneimine (PEI) cellulose plate. Thin-layer chromatography was performed against 1:1 ratio of 1M LiCl and 1M HCOOH. The plate was then dried, exposed to film and corresponding radioactive spots were excised and counted. ATPase activity is determined from the amount of [p^{32}]-ADP and [p^{32}]-AMP generated from [α-p^{32}]-ATP, i.e. the percentage of ATP hydrolyzed calculated as [ADP+AMP]/[ATP+AMP+ADP]X100%. Background ATP hydrolysis lacking purified gp96 or hsp70 was subtracted.

Phosphate labeling and immunoprecipitation

Labeling cells with p^{32} was done according to Downward et al (Downward et al 1990). Immunoprecipitation was carried out using Townsend protocol (Townsend et al, 1990). To elute nucleotides, immunoprecipitates were incubated with 2 mM EDTA, 2 mM DTT, 0.2% SDS, 0.5 mM ATP, 0.5 mM ADP and 0.5 mM AMP at 68°C for 20 min. Separation of eluted nucleotides was on PEI-cellulose plates chromatography.

SDS-PAGE, Western blot, silver staining and immunoaffinity protein G column

Western blot was done according to HRP color developing system (Bio-Rad). Silver staining and protein G immunoaffinity chromatography were carried out using standard protocols.

Peptide extraction and separation

Isolation of peptides from purified gp96 was done by the acid extraction technique (Van Bleek and Nathenson, 1990). The low molecular weight materials were then enriched by separating out the high molecular weight proteins using centricon-10 spin column (MW cut off: 10,000 dalton, Amicon) according to the manufacturer's instructions. The low MW material isolated from gp96 was analyzed by directly injecting into HPLC (Applied BioSystems) and further resolved using C18 column and acetonitrile gradient with flow rate of 1 ml/min (Buffer A, 0.05% TFA; buffer B, 100% acetonitrile, the gradient is linear from 0% buffer B to 100% buffer B). Fractions were collected at 1 ml/fraction/min.

Acknowledgement

We thank Nathalie Blachere for purification of the macaque gp96, Anya Harry for help with the *in vitro* ATP binding experiments and Dr. Reza Green for critically reading the manuscript. This work was supported by NIH grant CA44786. P. K. S. is an Investigator of the Cancer Research Institute, New York, and an Irma T. Hirschl Scholar.

CHAPTER 3. MHC CLASS I MOLECULE IS ASSOCIATED WITH GP96/GRP94, A CHAPERONE LOCALIZED IN THE LUMEN OF THE ENDOPLASMIC RETICULUM*

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(*This paper has been submitted for publication)

Summary

Stress-induced protein gp96/grp94, a major component of the lumen of the endoplasmic reticulum, is shown to associate with MHC class I molecules in murine and human cells. Immunopurified MHC class I preparations contain gp96 as well as the Class I-binding protein p88/calnexin. Together with our recent demonstration that gp96 is a peptide-binding protein and an ATPase, and the functional studies which show that antigenic peptides are associated with gp96, this observation argues that gp96 plays a role in facilitating assembly of the MHC class I-β2 microglobulin-peptide complex.

The MHC class I-β2m complex presents antigenic peptides derived from intracellular proteins to $CD8^+$ T lymphocytes. The assembly of this tri-molecular complex is believed to occur in the lumen of the endoplasmic reticulum (ER) (Nuchtern et al, 1989; Yewdell and Bennink, 1989; Lapham et al, 1993). Although purified MHC class I heavy chain, β2m and peptides can assemble *in vitro* (Chen and Parham, 1989; Silver et al, 1991), the assembly reaction in the ER is thought to be facilitated by one or more, so far unidentified, additional proteins (Alexander et al, 1989; 1990; Townsend et al, 1990; Levy et al, 1991; Gething and Sambrook, 1992). Candidates for this facilitating role may be expected to possess certain minimal properties such as, they should be able to bind and release peptides, perhaps in an ATP - dependent manner and should be able to associate with MHC class I molecules or Class I-β2 microglobulin complexes. We have recently shown that the heat shock protein gp96/grp94, a major component of the lumen of the ER, binds ATP and is an ATPase and is associated with a diverse array of peptides (Li and Srivastava, 1993). Gp96-peptide complexes have been previously shown be antigenically active and to elicit cellular immunity against tumors and viruses (Srivastava et al, 1986; Srivastava and Maki, 1991; Blachere et al, 1993). We demonstrate here that gp96 molecules associate with MHC class I during assembly of the trimolecular complex and that immunopurified MHC class I preparations contain gp96 as well as the Class I-binding protein p88/calnexin (Ahluwalia et al, 1992; Degen and Williams, 1991; Galvin et al, 1992; Hochstenbach et al, 1992; Wada et al, 1991). Our observations suggest a role for gp96 in facilitating assembly of the MHC class I with β2 microglobulin and peptides.

The initial studies were carried out in the RMA-S cell line. The assembly of MHC class I, β2m and peptides is inefficient in RMA-S because antigenic peptides are not transported into the lumen of the ER due to a mutation in one of the transporter proteins, TAP-2 (Attaya et al, 1992; Powis et al, 1991). We reasoned that it may be easier to identify assembly-facilitating proteins in RMA-S since the folding and assembly process of MHC class I in RMA-S is slow. RMA-S cells were metabolically labeled with ^{35}S methionine and the extracts were precipitated with anti - MHC class I and anti gp96 antibodies (Fig. 3.1). Antibody 28-14-8S, which recognizes the α3 domain of H-2D^b molecule (Townsend et al, 1990; Elliot et al, 1991) was observed to precipitate MHC class I and β2 microglobulin as well as a 96 kDa band (Fig. 3.1a, lane 1) which co-migrates with the 96 kDa band precipitated by the anti-gp96 monoclonal antibody (Fig. 3.1a, lane 2). Conversely, the anti-gp96 antibody precipitates a band which co-migrates with the MHC class I molecule precipitated by 28-14-8S in addition to the *bona fide* gp96. The anti-gp96 antibody does not appear to precipitate significant amount of labeled β2 microglobulin (discussed later). The identity of the 96 kDa band co-precipitated by 28-14-8S antibody as gp96 was confirmed by immunoblotting it with anti-gp96 antibody (Fig. 3.1b, lane 2). The identity of the MHC class I-like band precipitated by anti-gp96 antibody was tested by eluting the bands and re-precipitating them with 28-14-8S antibody (Fig. 3.1c). It was observed that the class I molecules eluted from the immunoprecipitations shown in Fig. 3.1a (*both lanes*) could be re-precipitated by the 28-14-8S antibody, but not by normal mouse serum.

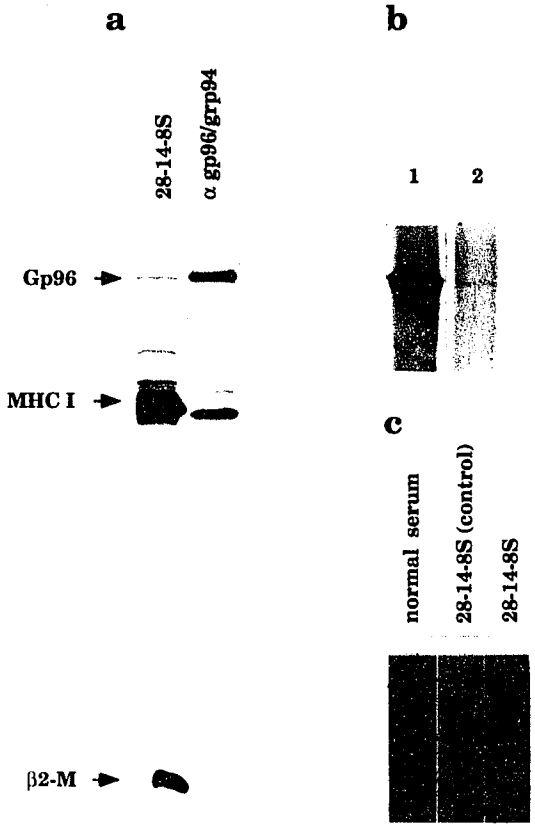

Figure. 3.1.

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Figure. 3.1. Co-immunoprecipitation of MHC class I with gp96. (a). RMA-S cells ($4x10^7$) were metabolically labeled and immunoprecipitated with 28-14-8S antibody (against α3 domain of $H\text{-}2D^b$) or a monoclonal antibody to gp96 (SPA-850, Stressgen). Immunoprecipitates were analyzed by 12% SDS-PAGE. **(b)** Samples shown in (a) were immunoblotted with antibody against gp96. Lane 1 corresponds to the 96 kDa material precipitated with antibody to gp96. Lane 2 is the 96 kDa molecule co-precipitated by the 28-14-8S antibody. **(c)** The bands in MHC I region of (a) were eluted and reprecipitated with 28-14-8S antibody. The middle lane represents control MHC class I proteins, while the right lane represents the class I molecules coprecipitated with gp96.

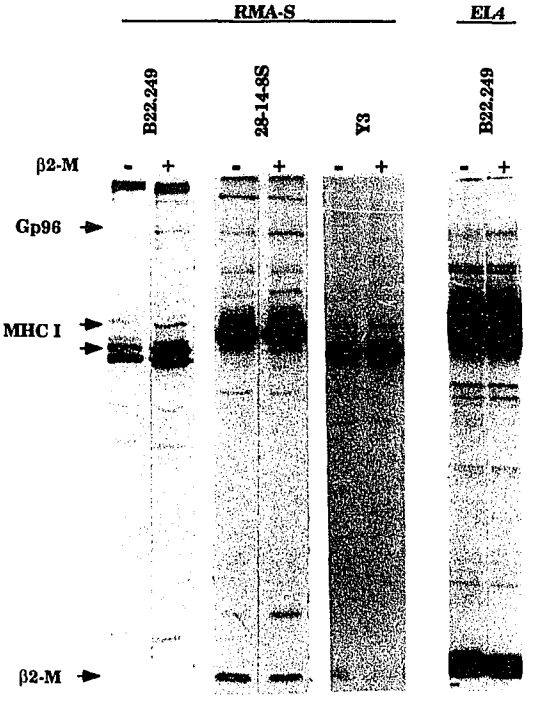

Figure. 3.2. Association of gp96 with MHC class I in the cell lysate is enhanced by preincubating cell lysates with human β2 microglobulin. Metabolically labeled RMA-S or EL-4 ($4x10^7$ cell lysate/lane) cells were lysed in presence or absence of 100 μM human β2 microglobulin. The first antibody (as indicated) was added 1 hour after cell lysis and allowed to incubate with the lysate overnight.

In contrast to the results with the α3 D^b-specific 28-14-8S antibody, precipitation of metabolically labeled RMA-S cell extracts with the B22.249 antibody (which preferentially recognizes the folded D^b, see Townsend et al, 1990), did not show co-precipitation of gp96 with MHC class I (Fig. 3.2). However, when the cell lysate was incubated with human β2 microglobulin for several hours on ice before immunoprecipitation, co-precipitation of gp96 with MHC class I could be clearly observed (Fig. 3.2). The requirement of pre-incubation of RMA-S cell lysates with β2 microglobulin for co-precipitation of gp96 is reminiscent of the observations of Townsend and his colleagues (Townsend et al, 1990), who showed a 105 kDa protein coprecipitating with H-$2D^b$ in RMA-S cell lysate only after pre-incubation of the lysates with β2 microglobulin. Two additional observations with gp96 mirror corresponding observations with p105 (Fig. 3.2): co-precipitation of gp96 with the α3-specific antibody 28-14-8S does not require pre-incubation with β2 microglobulin, although pre-incubation results in co-precipitation of slightly increased quantity of gp96. Secondly, gp96 co-precipitates with D^b, but not with K^b molecules, as judged by anti-K^b antibody Y3 (Townsend et al, 1990) (Fig. 3.2). These results suggest that gp96 is p105 and that the size difference is attributable to the difficulties in accurate mass determinations in this range by SDS-PAGE. Co-precipitation of gp96 with D^b molecules after pre-incubation of cell lysates with β2 microglobulin was also shown in antigen presentation-competent EL4 cells (Fig. 3.2).

The above results also bear on our observation that β2 microglobulin is seldom associated in any significant amount with the

class I heavy chain co-precipitated by the anti-gp96 antibody (Fig. 3.1a). It is possible that gp96 interacts with class I heavy chain as well as heavy chain-β2 microglobulin and the gp96-heavy chain-β2 microglobulin complex is present in the lysates, but is unstable in the conditions of washing and immunoprecipitation used by us. This view is supported by the observation (Fig. 3.2) that addition of excess human β2 microglobulin promotes detection of increased quantities of gp96-MHC heavy chain: the human β2 microglobulin is known to have a higher affinity than the mouse β2 microglobulin for mouse heavy chain and the complex with human β2 microglobulin is therefore more stable (Wei and Cresswell, 1992). The human β2 microglobulin is not visible in the immunoprecipitates because it is not labeled.

Association of gp96 and MHC class I was also demonstrated by co-purification of the two proteins. This approach circumvents the difficulty that metabolic labeling identifies only the newly synthesized proteins. H-2D^b molecules were purified from extracts of metabolically labeled 2X10^9 RMA-S cells, by immunoaffinity chromatography with the B22.249 antibody. The left lane of Figure 3.3 shows the results of autoradiography of the SDS-PAGE profile of the purified D^b. This material was immunoblotted and the blots probed with anti-gp96 antibody. A gp96 band can be clearly detected in the immunoaffinity purified D^b preparation (Fig. 3.3). Immunoblotting also revealed the presence of p88/calnexin in this preparation (Fig. 3.3). In order to test the specificity of this association, the blots were probed with antibodies against a moderately abundant ER protein ERp72 (Mazzarella and Green, 1987) and also against an abundant cytosolic protein hsp70. Neither of these proteins were detected in the class I preparation.

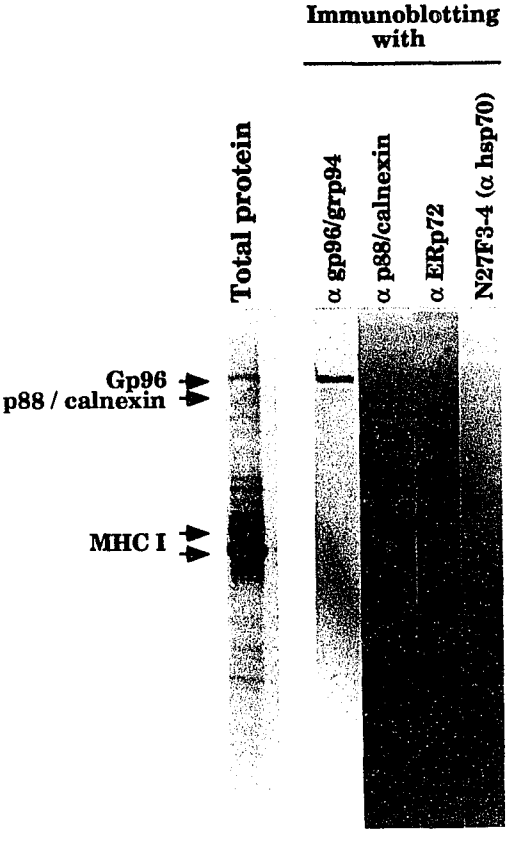

Figure. 3.3. Copurification of gp96 with MHC class I. H-2D^b molecules were purified from the detergent lysate of the metabolically labeled 2X10^9 RMA-S cells (Parham, 1982). Left lane shows the autoradiogram of the SDS-PAGE of immunopurified MHC class I preparation. The purified samples were immunoblotted with antibodies as indicated. Total cell lysate was used as a positive control for each antibody and found to be positive (not shown).

Two aspects of co-precipitation of gp96 with MHC class I are to be noted. Firstly, while significant amounts of class I molecules co-precipitate with gp96 by anti-gp96 antibody, relatively small quantity of gp96 co-precipitates with MHC class I by anti-class I antibodies. This is seen dramatically in case of human T2 cells, where abundant class I molecules are co-precipitated with gp96, while no gp96 band is seen on precipitation with the anti-human class I antibody W6/32 (Fig. 3.4a). Identity of the class I co-precipitated with gp96 is confirmed by eluting the class I band and re-precipitating the eluate with W6/32 (Fig. 3.4b). The asymmetry between the quantities of gp96 co-precipitated with class I and class I co-precipitated with gp96 suggests that only a small proportion of MHC class I molecules is bound to a small proportion of gp96 at any given time. This proportion may either represent a fraction of the total class I, or more likely, a particular conformational species of class I. As gp96 binds antigenic peptides and is an ATPase (Li and Srivastava, 1993; Blachere et al, 1993), only the freshly transported MHC class I molecules which are *in the process of receiving peptides* from gp96, will be found associated with gp96. This would indeed be a small proportion of the total class I binding to a small proportion of gp96 at a given time. However, this by itself may not completely account for the observed asymmetry. At least part of the asymmetry in co-precipitation of gp96 and MHC class I may also result from the conformational heterogeneity of class I because of the folding process and the fact that a given anti-class I antibody precipitates only a proportion of class I molecules due to the fine specificity of the anti-class I antibodies. Gp96, on the other hand, associates with any class I heavy chain.

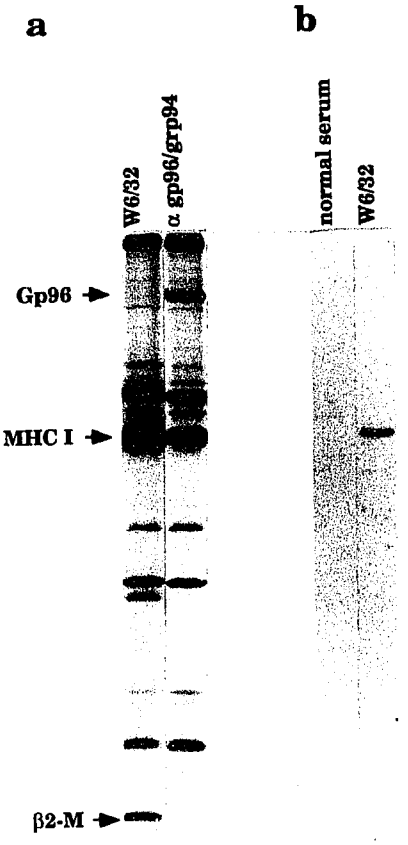

Figure. 3.4.

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Figure. 3.4. MHC class I is associated with gp96 in T2 cells. (a) Metabolically labeled T2 cells ($3x10^7$/lane) were lysed and precipitated with either W6/32, an antibody against folded HLA-A, B and C or a monoclonal antibody against gp96. **(b)** Material migrating around MHC I region in anti-gp96 antibody precipitation lane in (a) was eluted and incubated with 50 μM human β2 microglobulin overnight in 0.5% NP-40 containing buffer (as described in the legend to Fig. 3.1c). The reconstituted materials were reprecipitated either with normal mouse serum or W6/32.

The second aspect of gp96 - MHC class I interaction has to do with the interaction of gp96 with D^b but not K^b molecules (Fig. 3.2) and may result from differences in the folding and transport rates of the individual class I molecules (Williams et al, 1985; Alexander et al, 1989; Owen et al, 1980). Preferential association of some alleles with gp96 may lead to a more efficient folding and the fact that H-$2D^b$ molecule can travel to cell surface without β2 microglobulin is consistent with such a possibility (Williams et al, 1988; 1989).

Assembly of the MHC class I heavy chain-β2 microglobulin-peptide complex requires two discrete steps. One is establishment of the peptide-binding groove of the heavy chain (Bjorkman et al, 1987a; 1987b), which occurs almost co-translationally *in vivo*. The second step is peptide loading, which has been shown to be rate-limiting (Townsend et al, 1990; Elliot et al, 1991). The proteins associating with MHC class I, such as p88/calnexin and gp96, can be expected to play important roles in both these steps. The p88/calnexin is thought to primarily hold the class I heavy chain in the ER so that assembly can occur (Degen et al, 1992). Since gp96 has been shown to chaperone antigenic peptides, binds ATP and is an ATPase (Srivastava et al, 1986; Li and Srivastava, 1993; Blachere et al, 1993), it is logical to propose that it plays a critical role in peptide loading. Gp96 and p88/calnexin would thus perform distinct and complementary roles in assembly of the trimolecular MHC class I-β2 microglobulin-peptide complex. Crucial questions regarding this assembly remain unanswered. Which steps, if any, in the assembly process are ATP-dependent? Do other ER luminal proteins such as BiP/grp78 play a role in assembly? What is the sequence of association/dissociation of individual members of the trimolecular

complex with gp96 and p88/calnexin and with respect to the final 'sizing' of antigenic peptides? These questions clearly require much more resolution. Nevertheless, the observations that the heat shock protein gp96 is an ATPase, chaperones antigenic peptides and interacts with MHC class I, provide a major new clue to understanding of MHC class I folding and assembly.

CHAPTER 4. DISCUSSION

Section #4.1. Summary of results.

The biochemical basis of immunogenicity of gp96 as a tumor rejection antigen has been explored in this work. The major findings are:

(1) gp96 is a Mg^{2+}-dependent ATPase. The activity has a Km of 8 μM and the turn over rate of 0.08 mole/min/mole;

(2) The ATPase activity of gp96 is stimulated by misfolded protein such as dephosphorylated casein but not by peptides, suggesting that gp96 ATPase activity is regulated by gp96-protein interaction rather than gp96-peptide interaction;

(3) gp96 is associated with MHC class I in human T2 cells as well as murine RMA-S and EL-4 cells;

(4) Immunoprecipitation by conformation-dependent antibodies against MHC class I indicates that gp96 is preferentially associated with unfolded and thus peptide-free MHC class I;

(5) Peptides can be extracted from purified gp96, a property of gp96 similar to that of the MHC molecules.

The above observations support the suggestion that the tumor-specific immunogenicity of gp96 is not against gp96 *per se*, but against gp96-associated peptides. They allow us to propose further that gp96 is a protein chaperone for MHC class I. The existence of such a chaperone has been proposed for many years, but none has been identified so far.

Section #4.2 Case for an ER luminal chaperone that facilitates MHC class I-peptide assembly.

The site of MHC class I-peptide assembly is assumed to be the lumen of the ER, on basis of the effect of brefeldin A on the class I restricted antigen presentation (Nuchtern et al, 1989; Yewdell and Bennink, 1989). It has been confirmed recently that class I molecules retained in the ER associate with naturally processed peptides at about equal efficiency to wide type class I (Lapham et al, 1993).

The assembly of MHC class I-peptide complex is a complex process whose elucidation is a major challenge. Identification and characterization of a number of cell lines defective in the assembly of MHC class I-peptide complex indicates that the assembly is a multi-step process. Some of the possible reactions have begun to be revealed albeit without knowing anything about the catalytic factors involved. Fig. 4.1 is taken from Elliott's recent review on MHC class I-peptide interactions with slight modifications to emphasize the lack of knowledge on the catalytic factors (Elliott, 1991). All of the steps shown in the figure have been observed to happen in solution reaction using a model MHC class I molecule, H-2D^b (Elliot, 1991; Elliott et al, 1991; Townsend et al, 1989; Townsend et al, 1990).

Several pieces of critical information have been learned using this system, which are applicable in general to the assembly of diverse MHC class I molecules.

First, although class I molecule assembly can be dissected into multiple reactions, β2 microglobulin binding, peptide association and heavy chain assembly are linked, and cooperative phenomena.

Second, the folding of the α3 domain of the class I heavy chain molecule can occur in the absence of β2 microglobulin and peptides. More importantly, α3 domain folding is the prerequisite for conformational maturation of the α1 and α2 domains.

Third, the major pathway of class I assembly is thought to be through K0 → K1 → K2 → K3 → K4 (Fig. 4.1). Peptides longer than optimal length can associate with MHC class I-β2 microglobulin complex (K3), but the association constant (ka) of this step is low. Peptide alone can bind heavy chain in the absence of β2m (K5), however such peptides have to be in optimal length (8-9 mer).

Finally, most of the reactions described in the Fig. 4.1 are reversible and tends to reach equilibrium in physiological conditions. Therefore, increasing the absolute concentrations of either heavy chain, β2m or peptides can accelerate the assembly process.

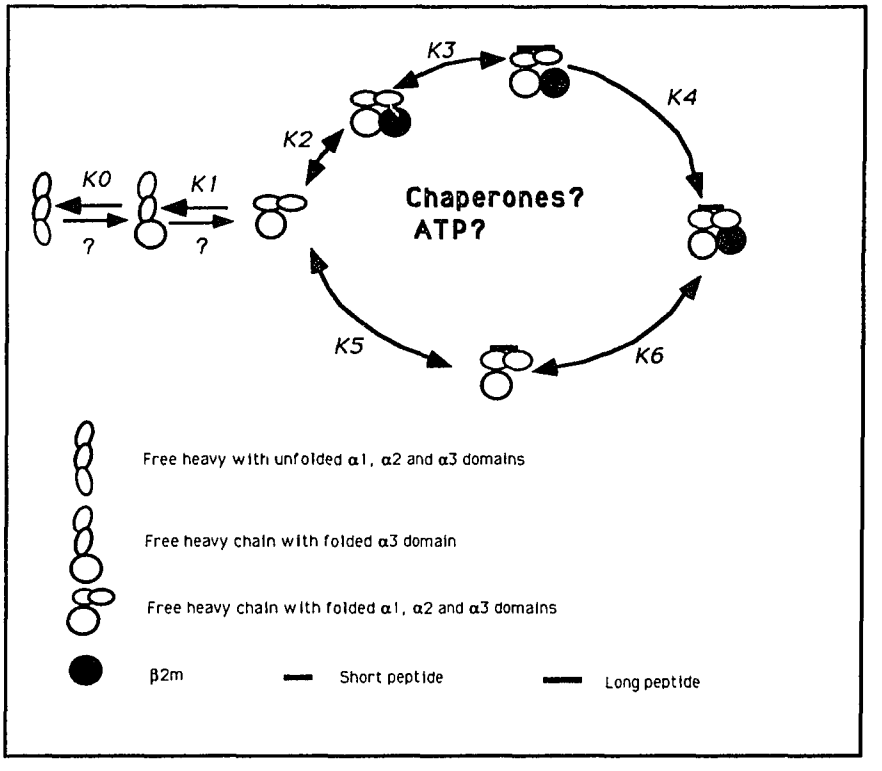

Figure 4.1. Biochemical events leading to the association of peptides with MHC class I-β2 microglobulin complex. See text for details. Modified from Elliot, 1991.

It is worthwhile to identify the caveats in the above model of MHC class I-peptide assembly. The above information is based entirely on *in vitro* systems, which are artificial. In most cases, class I heavy chain, β2 microglobulin and peptides are arbitrarily mixed and their interactions followed. Clearly, the folding reaction in RMA-S cell lysate developed by Townsend and his coworkers may not accurately reflect what occurs going on *in vivo*. Furthermore, the site of MHC class I-peptide assembly is known to be in the ER lumen. But there is no information on whether the assembly occurs in a specific unidentified subcompartment of the ER or merely in the rough ER. This point is important because the microenviroment where the assembly occurs might dictates crucial factors such as peptide concentration, ionic composition etc., which may be essential for proper assembly. Lastly, most of the molecular processes involving protein folding in the ER are poorly understood.

The complexity and efficiency of MHC class I-peptide assembly *in vivo* argues for a role of protein chaperone(s) in the process. The involvement of chaperone(s) was suggested clearly by the discovery that HLA-B27 and HLA-A2 assembly in microsome system is strictly ATP-dependent (Lévy et al, 1991; Kvist and Lévy, 1993). In such experiments, class I heavy chain is translated in reticulocyte lysate plus microsomes. The translation mixture was depleted of ATP by treatment with apyrase, an enzyme hydrolyzing ATP to ADP and AMP. Peptides were then added following the blockage of translation. The efficiency of assembly is judged by immunoprecipitation with W6/32, an antibody recognizing only fully assembled HLA class I. It was found that no assembly occurs after ATP depletion.

There are three additional clues to suggest the presence of catalyst(s) in MHC class I-peptide assembly. One is the discrepancy between the kinetic rate of MHC class I assembly between *in vivo* and *in vitro*. MHC class I heavy chain, β2 microglobulinm and peptide, when in biochemically homogenous form, can form a competent tertiary complex *in vitro*. However, MHC class I-β2 microglobulin-peptides assembly reactions *in vitro* described so far are generally inefficient (Chen and Parham, 1989; Silver, et al, 1991). It takes more than 24 hours to achieve appreciable level of assembly. This is far slower than the rate of assembly *in vivo*. Kinetics study indicated that the assembly process can be as short as 45 minutes beginning from the *de novo* synthesis of MHC class I (see review by Yewdell and Bennink, 1992).

The second clue is the fact that different alleles of MHC class I travel to cell surface at different rates (Alexander et al, 1989; Williams et al, 1985; 1988). More importantly, the differential rate of transport correlates with the folding of peptide-binding domain but not other regions nor the posttranslational glycosylation (Alexander et al, 1990; Williams et al, 1988; Owen et al, 1980). This means that different alleles of MHC class I, although homologous in sequence, have different folding efficiency. Since it is difficult to explain this divergence by some subtle difference in sequence, it has been postulated that there exists a folding machinery in the cell which regulates the rate of MHC class I folding/assembly.

Lastly, the existence of a chaperone protein for charging peptides to MHC class II has been reported (Lakey et al, 1987; Vanbuskirk et al, 1989; 1991; DeNagel and Pierce, 1992). By analogy, it is reasonable to assume a similar protein for MHC class I-peptide assembly.

Section #4.3. Molecules shown to be associated with MHC class I

The straightforward approach to identify accessory factors involved in the MHC class I folding and assembly with β2m and peptides is to look for proteins associated with class I. A number of such proteins have been described (Table 4.1).

There are two important steps in the trimeric formation of MHC class I heavy chain, β2m and peptide. Both steps are likely to be facilitated by chaperone-like proteins. One is the establishment of the peptide-binding groove of the heavy chain (Bjorkman et al, 1987a and 1987b), which occurs almost co-translationally *in vivo*. The second is the peptide loading step, which has been shown to be rate-limiting (Townsend et al, 1990; Elliot et al, 1991). The proteins associating with MHC class I are likely to play important roles in both of these steps, although it is not entirely clear at this point whether these MHC class I associated proteins are artifacts as a result of conformational change during folding and assembly process.

A brief discussion of each of these molecules is in order. P88 has been shown to be identical to calnexin, an ER integral membrane protein which is one of the components of the receptor for signal recognition particle (Ahluwalia et al, 1992; Wada et al, 1991; Galvin et al, 1992). P88 is also the same molecule as IP90 which binds unassembled T cell receptor (Hochstenbach et al, 1992). The association of p88 with class I heavy chain is nonselective with respect to the folding and assembly state as long as heavy chain is still in the ER (Degen et al, 1992). MHC class I is a glycoprotein targeted into the constitutive secretory pathway.

The function of p88 could thus simply be to physically hold the heavy chain in the ER so that assembly can take place.

The nature of p105 (105 kDa protein) is not defined, but work presented here strongly suggests that it is identical or similar to gp96. Since gp96 has been shown to carry antigenic peptides, it is logical to propose that it plays critical role in the peptide loading step of MHC class I-peptide assembly (see section #4.5).

So far grp78 has only been reported to associate with MHC class I in Daudi cells where there is no β2 microglobulin. Since heavy chain is not folded well without β2m, the MHC class I heavy chains in Daudi cells may simply represent misfolded proteins. Such interactions thus are consistent with the idea that grp78 plays a general role in protein folding. Whether grp78 is facilitating MHC class I folding *per se* remains to be seen.

P33 is one form of invariant chain molecules which are normally associated with MHC class II in the ER and are proteolyticly removed in endosome when MHC class II encounters antigenic peptide. So far only the transfected D^b molecules in T2 cells have been observed to associate with p33. The significance of this association is thus difficult to assess.

P24 associates with HLA-B5 and H-$2K^b$ in T2 cells. The nature of this protein is not known.

Table 4.1. Proteins associated with MHC class I.

Proteins	Alleles of MHC class I	Cells	Reference
p88/IP90	HLA-A, B, C H-2K, D, L	all examined	Degen and Williams, 1991 Hochstenbach et al, 1992
gp96	H-2D^b, HLA class I	T2, EL-4, RMA-S	This work (chapter 3)
p105	H-2D^b	RMA-S	Townsend et al, 1990
grp78	HLA-A, B, C	Daudi	Degen et al, 1992
p33	H-2D^b	T2 transfectant	Cerundolo et al, 1992
p24	HLA-B5, H-2K^b	T2 transfectant	Cerundolo et al, 1992

Section 4.4. Gp96 as a candidate for chaperone for MHC class I.

A role of protein chaperone(s) in the folding of MHC class I and the assembly of MHC class I with peptides has been postulated for a number of years (see above). Such proteins have not been identified. However, recent developments in MHC class I folding/assembly suggest that such molecule(s) should have the following minimal properties.

- *It should bind or should be in close proximity to MHC class I in vivo.*
- *It should bind to ATP or perhaps hydrolyze ATP since the assembly process is ATP-dependent.*
- *It should be in the lumen of the ER where the assembly occurs.*
- *Such a molecule should also bind peptides if it plays a specific role in the peptide loading step of MHC class I-peptide assembly.*

Based on the above criteria, gp96 is a good candidate for chaperoning MHC class I *in vivo*. This role for gp96 generates a number of testable predictions. If the hypothesis that gp96 binds peptides and facilitates MHC class I-peptide assembly is correct, gp96 should bind any peptides especially viral peptides that are processed and transported into the ER of virus-infected cells. Thus gp96 purified from virus-infected cells should bind peptides derived from viral proteins. This prediction has turned out to be correct. It was shown that immunization with gp96 purified from virus-infected cells resulted in the generation of MHC class I-restricted virus-specific CTLs. This phenomenon has been so far

observed in three different systems; influenza virus and SV-40 virus (Blachere et al, 1993), and Friend leukemia virus (W. Chen, N. Blachere, M. Cheever and P. K. Srivastava, personal communication).

A second prediction of this hypothesis is that the peptides associated with gp96 should be antigenic. This was tested in our laboratory by stripping peptides from gp96 purified from influenza virus infected target cells, and pulsing the syngeneic virus-uninfected target cells with the stripped peptides. If gp96 carries influenza virus specific peptides, one would expect the sensitization to be successful. Preliminary results in our laboratory indicates that this is indeed the case.

Table 4.2 is the list of the total evidence to date supporting the role of gp96 in the assembly of MHC class I-peptide complex. While it seems clear that gp96 chaperones MHC class I, the molecular details are still speculative. We favor the model that gp96, by virtue of its ability to hydrolyze ATP, to bind peptides and MHC class I, facilitates peptide loading of MHC class I molecule. The net effect is to promote and stabilize the peptide-bound MHC class I-β2 microglobulin complex. In this context, gp96 would be a *bona fide* chaperone for MHC class I (see next section).

Table 4.2. List of evidence supporting the role of gp96 in antigen presentation by MHC class I

- Immunizations with gp96 derived from tumor cells but not normal cells elicit protective tumor-specific immunity to the tumor from which it is derived (Srivastava et al, 1986; Udono and Srivastava, 1993; Blachere et al, 1993).
- Gp96 purified from virus infected cells can immunize and elicit virus-specific CTLs in a number of systems (influenza, SV40, Friend leukemia virus) (Blachere et al, 1993; W. Chen, N. Blachere, M. Cheever and P. K. Srivastava, personal communication).
- Purified gp96 possesses heterogeneous endogenous peptides (Li and Srivastava, 1993; this work, chapter 2).
- Gp96 is the single most abundant soluble protein in the lumen of the ER, where the assembly of MHC class I, β2 microglobulin and peptides takes place (Booth and Koch, 1989).
- Gp96 contains ATP-binding cassettes, binds with ATP and is a Mg^{2+} dependent ATPase, consistent with the ATP-dependent process of MHC class I-peptide assembly (Li and Srivastava, 1993; this work, chapter 2).
- Gp96 interacts with MHC class I (this work, chapter 3).
- Similar to other potential components of the MHC class I assembly system (heavy chain, TAP, proteasome), expression of gp96 is up-regulated by γ-IFN (B. Rubin and P. K. Srivastava, personal communication).

Section #4.5. A model for the role of gp96 in the MHC class I folding/assembly.

A model of MHC class I folding/assembly emerges from these studies (Figure 4.2). Free heavy chain associates with p88 immediately after synthesis (stage I, Fig. 4.2) and remain associated until class I molecule egresses the ER. The luminal peptide-bound gp96 begins to associate with class I heavy chain (stage II and III). Simutaneously, β2 microglobulin joins the complex (complex IV). The trimeric interaction among gp96, heavy chain and β2 microglobulin triggers the ATP hydrolysis by gp96, leading to conformational alteration of gp96. Consequently, antigenic peptides are released from gp96 and transferred to the heavy chain-β2 microglobulin complex (stage IV to stage V). Fully folded and peptide-bound MHC class I molecule dissociates from both p88 and gp96 (stage V), and travels to cell surface.

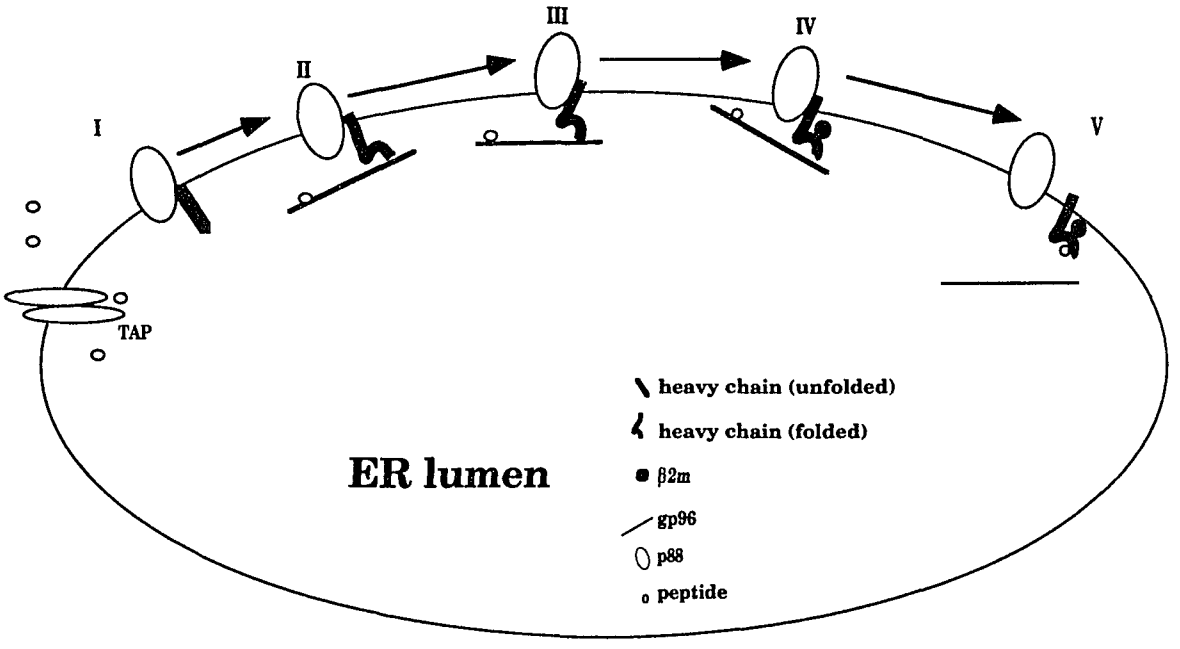

Figure 4.2. Gp96 chaperones MHC class I. Gp96 interacts with both the free heavy chain of the MHC class I and the peptide-unbound form of the MHC class I-β2m complex. The oligomeric interaction is presumed to stimulate the ATPase activity of gp96, leading to the transfer of peptides to MHC class I.

This model may be oversimplified as a number of important details are still unknown. For example, it is not clear which steps are truly ATP-dependent. What is the role of grp78/Bip? Could grp78 be compensating for the role of gp96 or having a more unique function? It is possible that only peptide-bound gp96 engages with MHC class I, since we do not find significantly increased binding of gp96 to MHC class I in RMA-S cells compared to EL-4 cells. How is the efficiency of folding/assembly ensured? Nevertheless, the observations that gp96 binds peptide, hydrolyses ATP and interacts with MHC class I provides an important clue towards understanding the fascinating nature of MHC class folding/assembly.

Section #4.6. Explanation for the role of gp96 as a TRA

Gp96 molecules were originally identified by transplantation assays in the search for a tumor rejection antigen, a molecule which was defined in this context of its abilities to immunize mice against tumor challenges. It has become evident now that the tumor-specific immunogenicity of gp96 is not against gp96 *per se* but against gp96-associated peptides. This implies that tumor cells must contain a peptide repertoire which is different from normal tissue. Such implication is reasonable in view of the carcinogen-induced origin of the tumors under study. We are currently working on the structure of antigenic peptides derived from gp96 purified from tumor cells. Once this is achieved, we should have some idea as to the nature of peptides delivered to immune system by gp96 to result in such profound tumor rejection.

We have not addressed how peptides are transferred to MHC class I after gp96 immunization. This is not a trivial issue since the immunization is so efficient and gp96-peptide complexes are obviously exogenous antigens. But there are indications that a group of macrophage-like cells in normal spleen can present exogenous antigen to MHC class I (Pfeifer et al, 1993; Rock et al, 1993). Interestingly, the primary phase of gp96 immunization is dependent on the macrophage (Udono and Srivastava, 1993c).

Section #4.7 Future directions

Although the evidence supporting the role of gp96 in antigen presentation by MHC class I is substantial (see Table 4.2), it must be emphasized that, at this point, our collective data can still be explained by entirely different views. Gp96 is a HSP and has been shown to bind to misfolded proteins such as the aberrant forms of glycoprotein b of the herpes simplex virus 1 (Navarro et al, 1991), immunoglobulin heavy chain (Melnick et al, 1992) and MHC class II (Schaiff et al, 1992). The fact that gp96 binds peptides may therefore simply happen post-cell lysis, which merely represent the promiscuous nature of substrate binding of gp96. Moreover, the association of gp96 with MHC class I molecule may have nothing to do with peptide-loading, and the gp96-associated MHC class I molecule could be just misfolded proteins. Finally, the functional requirement of gp96 to be an ATPase is not clear. There is still room to speculate that the ATPase activity of gp96 is dictating gp96 for other purpose such as of proteolyticly trimming

peptides in an ATP-dependent way, rather than that of physically loading peptides to MHC class I.

Thus more effort has to be spent in finally pinpointing the above considerations and to eventually prove that gp96 plays important role in antigen presentation by MHC class I - a hypothesis of obvious theoretical and practical significance. The following are some representative experiments for such purpose.

What is the nature of gp96-associated peptides?

MHC class I associates preferentially with nonameric peptides. It is not clear however how peptides are produced, transferred and finally loaded to MHC class I. One school of thought is that precursor peptides of longer than 9-mer are initially produced and bound to MHC class I. As MHC class I-peptide complex matures, the longer peptides get trimmed by some unknown protease(s) (Rotzschke and Falk, 1991). The structural definition (length, hydrophobicity, sequence) of gp96-associated peptides is essential for us to know whether gp96 is playing a role in sorting and transferring peptides to MHC class I.

Two systems can be adopted for such purpose. One is developed by Rammensee and his colleagues, which allows analysis of total peptide pool (Rotzschke and Falk, 1991). Total gp96-associated peptides can be isolated by acid extraction, subjected to Edman degradations, and the relative signal of a particular amino acid at each position can be calculated. In this manner, the average length of the total peptides and preferential usage of amino acids at a given position can be estimated.

The resulting information is then compared with the structural motifs of MHC class I-associated peptides.

The second system is to isolate and sequence gp96-associated peptides individually. The method is similar to characterization of peptides isolated from MHC class I and MHC class II, which includes acid extraction, HPLC, chemical sequencing or/and mass spectrometry analysis. Such effort will tell us what kind of peptides are associated with gp96, and what protein these peptides are derived from.

Is the association of gp96 with MHC class I physiological?

Gp96 has been shown to bind MHC class I by coimmuno-precipitation and co-purification strategy. More experiments are required to clarify which subsets of MHC class I (in terms of allele specificity, conformational heterogeneity etc.) are associated with gp96. The following experiments will be helpful to address this point.

A. Endo H sensitivity experiment.

Both gp96 and MHC class I are glycosylated proteins. A subset of gp96 molecules can travel to cell surface (Srivastava et al, 1986; A. Altymeyer and P. K. Srivastava, personal communication). As glycosylated proteins travel through cis Golgi, the carbohydrates get processed so that the covalently-linked carbohydrates become resistant to the cleavage by endoglycosidase H. Thus by determining whether gp96 and MHC class I are sensitive to endo H when they are in the complexed form, one can answer where and when does the association of gp96 with MHC class I occur.

B. The effect of peptides on the binding of gp96 to MHC class I.

The association of gp96 with MHC class I is increased when the RMA-S cell lysate is incubated with human β2m. The basis of this phenomenon is not clear. One likely possibility is that β2m stabilizes peptide-free MHC class I and only peptide-free MHC class I binds gp96. Such speculation can be tested by incubating cell lysates with allele-specific antigenic peptides and monitoring the association of gp96 with MHC class I.

C. Demonstration of the association of gp96 with MHC class I by *in vitro* translation.

An alternative approach to show the association of gp96 with MHC class I is to co-translate the mRNA of gp96 and MHC class I in microsomal system in the presence or absence of peptides. Such experiment is feasible only if gp96-free microsomes are used. Fortunately, one can deplete gp96 from microsome by alkaline treatment (Nicchitta and Blobel, 1993).

What is the significance of the ATPase activity of gp96?

The assembly of MHC class I in the ER is ATP-dependent. But it is entirely unclear which step requires ATP. There are reasons to believe that some other molecules are involved in the assembly process of MHC class I heavy chain, b2m and peptides, which complicates the analysis. For example, an integral ER membrane protein, p88 has been shown to bind many nascent polypeptides including class I heavy chain in the ER. Another ER lumen protein, grp78 has been shown to be able to bind

peptides *in vitro*. Currently, there are no functional evidence that these two proteins are involved in the MHC class I folding process.

Results presented in this thesis suggest strongly that MHC class I is probably a physiological substrate of gp96. But direct evidence has to be obtained that the ATPase activity of gp96 is functionally required to transfer peptides from gp96 to MHC class I. This point can be partially addressed *in vitro* by looking if purified MHC class I can stimulate the ATPase activity of gp96. Of note, the ATPase activity of other HSPs such as HSP60 and HSP70 can be dramatically stimulated by their substrates. This feature has provided a simple biochemical screening method for looking for substrates or other interacting components of HSP. For example, the cooperative role of DnaK with DnaJ and GrpE was firstly indicated by the fact that DnaJ ATPase activity can be stimulate up to 50 fold by the presence of both DnaJ and GrpE (Liberek et al, 1991).

If purified MHC class I such as H-2D^b indeed stimulates the ATPase activity of gp96, it remains to be determined if peptides are released from gp96 as a results of the interaction between gp96 and MHC class I.

Does exogenous gp96 facilitate the folding and stability of MHC class I molecule in vitro?

One can address this question using RMA-S cell system developed by Townsend and his colleagues (Townsend, et al, 1989; Elliot et al, 1991). Basically, RMA-S cells have an inefficient mechanism of antigen presentation through class I pathway. This was demonstrated to be at least partially due to point mutation of TAP-2 gene. Because of this defect

in class I assembly, RMA-S cells have much more unfolded or partially folded (thus unstable) class I molecules both on the cell surface and in the whole cell lysate than the parental wild type RMA cells. It was observed that class I assembly can be accelerated by adding exogenous peptides and β2m even in the detergent cell lysates. One can deplete endogenous gp96 from the cell lysate or add large excess of exogenous gp96 into it. The effect of such manipulation on the assembly of MHC class I and peptides can then be monitored. For example, is the reaction driven by gp96 ATP-dependent? Does gp96 provide peptides, facilitate MHC class I folding or both? What will be the result if one use denatured gp96 or empty gp96 by removing associated peptides?

Answers to the above questions will surely advance our understanding of the function of gp96 in antigen presentation. Of note, the details of antigen presentation are still poorly understood. For example, we do not know how antigenic peptides are generated. It is not clear whether the putative peptide transporter is transporting peptides or other cofactor(s) needed for MHC class I-peptide assembly. The role of chaperone proteins in peptide sorting has not been examined.

Thus work presented here is just another step towards the final understanding of the functions of gp96. The interests in the role of gp96 in MHC class I folding/assembly will continue, although it is almost certain that the function of gp96 is not restricted to antigen presentation (Nicchitta and Blobel, 1993).

Efforts should be made to search for cell lines which are defective in antigen presentation, as such line will reveal additional steps in this process. It is also important to look for novel immunosuppressive drugs and look into the effect of such reagents on antigen presentation by MHC

class I. A recent study has shown that the immunosuppressive effect of deoxyspergualin is mediated by its specific binding with hsp70 (Nadler et al, 1992). It will be interesting to know the functional consequences of such interaction including the effect on antigen presentation.

APPENDIX

Antigen presentation by MHC class I

Interactions between T lymphocytes and their targets or other immune cells are controlled by the products of the genes of major histocompatibility complex. Antigen presentation refers to a cascade of events starting from the generation of peptides, the MHC-peptide assembly and transport of such a complex to the cell surface. Antigen presentation through MHC class I antigens is the focus of this discussion because gp96 is proposed to play a role in the presentation of endogenous antigenic peptides through this pathway. Rapid developments in this field recently have been due largely to the availability of the three critical experimental systems. First, cytotoxicity assay represents a highly sensitive method to evaluate the functionality of peptide-MHC class I complex. It is estimated that less than 200 functional MHC-peptide complexes on the surface of target cells can sufficiently sensitize the T cell (Christinck, et al, 1991) to execute target cells lysis. This can be easily detected by a standard Cr^{51}-release assay using Cr^{51}-labeled target cells. The second system is the establishment and characterization of several presentation-defective cell lines namely RMA-S in mouse and T2 in human. Studies on these cells have led to the discovery of a number of participating molecules in antigen presentation. The third powerful technique is the ability to crystallize the peptide-bound MHC class I complex, and to isolate and sequence endogenous (naturally processed) peptides associated with the class I molecules (for a

review, see Rötzschke and Falk, 1991). These techniques have provide a visual picture of the MHC class I-β2m-peptide trimeric complex.

How antigenic peptides are generated: a role of proteasome?

Most of the antigenic peptides associated with class I molecules are produced in the cytosol. The proteolytic machinery involved in the generation of the peptides has not been worked out. Two types of abundant proteases are ubiquitously present in the cytosol and nucleus in the eukaryotic cells. One is named as 20S proteasome or multicatalytic proteolytic complex (MPC) because it has multiple substrate specificities (Wilk and Orlowski, 1980; and also see reviews by Orlowski, 1990; Goldberg and Rock, 1992). The other is a larger complex called 26S proteasome. 26S proteasome has all the subunits of 20S proteasome and additional yet incompletely characterized subunits. While the structural relationship between 20S and 26S proteasomes is not clear, functional distinctions have been drawn: 26S but not 20S proteasome is responsible for the ubiquitin- and ATP-dependent proteolysis of intracellular proteins (Goldberg and Rock, 1992).

The first functional clue that ubiquitin-dependent proteolytic pathway is important for generating peptides presented by MHC class I was the report that a chimeric protein that consists of ubiquitin and viral protein was more efficiently presented to the MHC class I (Townsend et al, 1988). Recently, the importance of this pathway was further demonstrated by a study of a temperature-sensitive mutant cell that has a reduced level of ubiquitin-protein conjugation and protein degradation at 41°C (Michalek et al, 1993). It was shown that, although such cells

present an ovalbumin peptide synthesized from a minigene, they fail to present intact ovalbumin osmotically introduced into the cytosol.

The other interesting episode to link the function of proteasome to MHC class I-dependent antigen presentation came from the discovery that the genes encoding at least 2 components (LMP2 and LMP7, LMP stands for low molecular mass polypeptide complex) of proteasome-like complex LMP are physically linked with MHC gene complex (Kelly et al, 1991; Martinez and Monaco, 1991; Ortiz-Navarrete et al, 1991; Brown et al, 1991; Glynne et al, 1991). Moreover, the expression of those two genes can be up-regulated by γ-IFN. Since LMP is closely related to but different from 20S proteasome, Monaco proposed that LMP is a specialized subset of proteasomes involved in the class I MHC antigen presentation (Monaco, 1992; Goldberg and Rock, 1992).

However, other reports cast doubt on this assumption. The human lymphoblastoid B cell-derived mutant T2 cell line has a large homozygous deletion of the MHC class II region encompassing the genes for peptide transporters as well as the two genes for MHC-encoded LMP subunits. Two groups reported simultaneously that transfection of cDNAs of transporters but not the two proteasome subunits into T2 cells can restore fully the level of stable MHC class I expression and the ability of cells to present endogenous antigen (Momburg et al, 1992; Arnold et al, 1992). This indicates clearly that at least in lymphoblastoid cells MHC-encoded proteasome subunits are not essential for antigen presentation through MHC class I pathway.

Antigen processing in the ER?

At least one defined protease, signal peptidase, is present in the ER (YaDeau and Blobel, 1989; Dalbey and von Heijne, 1992; Sanders and Schekman, 1992). Peptides extracted from purified HLA-A2 molecules from T2 are mainly derived from signal peptides (Henderson et al, 1992; Wei and Cresswell, 1992). There are many experimental data indicating that more general types of protease(s) may be present in the ER. However, they have not materialized despite mounting evidence suggesting their presence (for reviews, see Klausner and Sitia, 1990; Bonifacino and Lippincott-Schwartz, 1991). The functions of those proposed protease(s) in the generation of class I-associated antigenic peptides are thus difficult to assess. In one report, it is shown that targeting angiotensin converting enzyme to the ER artificially by genetic techniques can boost the efficiency of antigen presentation through MHC class I (Eisenlohr et al, 1992). It has at least two implications: one is that peptide processing is probably necessary after import into the ER; second, it adds one more piece of evidence to the general assumption that the assembly of peptides with class I-β2m complex is in the ER.

Recently, Hammond et al (1993) reported that T2 cells can efficiently present the envelope protein of immunodeficiency virus 1 (HIV-1) to MHC class I. Since T2 cells do not have TAP proteins and envelope proteins are co-translationally targeted to the ER through signal sequence, such finding provides another clue that antigen processing in the ER is possible.

A role of TAP in peptide transport?

Studies in RMA-S and T2 have indicated that transport of peptides and MHC class I heavy chain into the lumen of the ER are separate events. The mechanism of peptide transport may involve two highly homologous proteins with a consensus ATP-binding cassette (ABC) in their sequence. This cassette is conserved among more than 30 members of "ABC" superfamily involved in transporting ions, saccharides, amino acids, peptides and even proteins (for review, see Juranka, et al , 1989). The two putative peptide transporters named as TAP-1 and TAP-2 (TAP stands for transporter-associated protein) (Monaco, 1992), are the membrane proteins of the rough ER, and are present in rat (Deverson, et al, 1990), mouse (Monaco et al, 1990) and human (Spies, et al, 1990; Trowsdale, et al, 1990). They exist largely as heterodimers (Kelly et al, 1992; Spies et al, 1992), and have the characteristic ATP binding cassettes protruding into the cytosol. The genes encoding TAP-1 and TAP-2 are colocalized within the MHC gene complex and their expression can also be upregulated by γ-IFN. Moreover, transfection of intact transporter cDNA into presentation-defective cell lines can restore almost fully the antigen presentation capacity (Powis, et al, 1991; Attaya et al, 1992; Spies and DeMars, 1991). Recently, Van Kaer et al (1992) provide *in vivo* evidence that TAP is important for generation of peptides. It is shown that mice disrupted in the TAP1 gene have drastically reduced levels of surface expression of MHC class I molecules and few $CD8^+$ T cells. However, it is still not clear whether TAP proteins transport peptides directly or some other cofactor(s) important for peptide loading

to the MHC class I. One report clouding this issue showed that peptide transport into microsome(s) *in vitro* is resistant to the pretreatment of microsome with proteinase K. Furthermore, again in the microsome system by the same report, transport is not dependent upon ATP and can occur successfully in the microsomes prepared from T2 cells where both alleles of the transporter genes are deleted (Lévy, et al, 1991). The controversies have not yet been reconciled successfully. It can be argued that there exists more than one mechanism for peptide transport, or perhaps, TAP is only present on a special subset of microsomes or the *in vitro* study does not reflect exactly the physiological situation.

TAP has also been suggested to be one of the selective mechanisms to determine what peptide will be ultimately presented, since TAP proteins have limited polymorphism (Powis et al, 1992). However, studies by exchanging human and murine TAP using transfection approach in TAP-mutant cell line have shown that TAP polymorphism has very limited influence, if any, on the types of peptides presented to the immune system (Yewdell et al, 1993; Lobigs and Mullbacher, 1993).

Peptide loading to the MHC class I-β2m complex in the ER lumen?

The assembly process is thought to occur in the ER or at least in the pre-Golgi compartment. This was based on the study of the effect of brefeldin A on the class I restricted antigen presentation (Nuchtern et al, 1989; Yewdell and Bennink, 1989). Lapham et al (1993) have recently provided direct evidence that naturally processed peptides associate with MHC class I molecules in an ER-like compartment. They showed that MHC class I molecules retained in the ER either by treatment with

brefedin A or by substituting the cytoplasmic domain with that of the adenovirus E3/19K glycoprotein associate with antigenic peptides with about equal efficiency to the wild type class I molecule.

A role of chaperone(s) in the MHC class I folding/assembly?

The mechanism of the assembly is unclear. Although biochemically purified MHC class I heavy chain (HC), β2m and peptides can assemble in vitro (Chen and Parham, 1989; Silver, et al, 1991), the possibility that the assembly reaction in the ER is facilitated by one or more chaperone proteins in an ATP-dependent manner is emerging (Alexander et al, 1989, 1990; Townsend et al, 1990; Levy et al, 1991; Gething and Sambrook, 1992). But up to now, the chaperone protein(s) participating in the process of MHC class I folding/assembly remain uncharacterized (see Section #4.2). P88, an ER membrane calcium-binding protein, was shown to form complexes with class I heavy chain during the early biogenesis of class I molecule (Degen and Williams, 1991). But the association of p88 with class I heavy chain is nonselective with respect to the folding and assembly state (Degen et al, 1992). MHC class I is a glycoprotein targeted into the constitutive secretory pathway. The function of p88 could thus simply be to physically hold the heavy chain in the ER so that assembly can take place (see more in section #4.4). Data presented here strongly suggest that gp96 is a chaperone protein for MHC class I folding/assembly, particularly in the peptide loading step. The role of another ER resident heat shock protein grp78 in class I assembly is not defined (Dobberstein, 1992; and also see section #4.4). Since MHC class I assembly is a multiple-step process including heavy

chain folding, β2m association, peptide selection and binding, and possibly peptide trimming, it is possible that the assembly machinery consists of multiple accessory factors.

Significance of MHC class I assembly on the cell surface?

Surface expression of MHC molecules is important for both positive and negative selection during lymphocyte repertoire development (Van Kaer et al, 1992: Ashton-Richardt et al, 1993). Thus, MHC class I assembly on the plasma membrane is likely to have functional significance. Studies using RMA-S cells indicated that all reactions leading to the maturation of the peptide-class I complex can occur at the cell surface although at lower efficiency (Elliot, 1991). The assembly on the cell surface likely happens *in vivo* based on another consideration: β2 microglobulin is secreted. However, the significance of MHC class I assembly on cell surface remains speculative.

Targeting exogenous antigens to MHC class I?

Exogenous antigens are generally phagocytized by antigen presenting cells, processed, and presented through MHC class II pathway. However, there is growing evidence to suggest that some exogenous antigens can be routed to MHC class I pathway.

By expressing an ovalbumin epitope inside the *E. coli* and using this as the source of exogenous antigen, Pfeifer et al (1993) showed that peritoneal macrophage can efficiently present this epitope to T cells. This

presentation is MHC class I-restricted, dependent on phagocytosis, and is resistant to cyclohexamide and brefeldin A.

Rock et al (1993) have also found that a group of macrophage-like cells in normal spleen can present exogenous antigen to MHC class I. The presentation is not inhibited by the inhibitors of thiol proteases such as leupeptin and antipain.

These reports suggest the presence of a novel vacuolar class I processing pathway for exogenous antigens. Such pathway may be present only in a limited cell types.

Sorting of peptides by MHC class I itself?

The efforts to crystallize class I molecules and to isolate the endogenous peptide pool associated with purified class I have led to the conclusion that the α1 and α2 domains of class I heavy chain form a peptide binding groove where it is occupied by peptides of about 9-mer in an extended conformation (Bjorkman, et al , 1987a, 1987b; Jardetzky et al, 1991; Madden et al, 1991; Zhang et al, 1992). Though the naturally processed peptides of MHC class I are generally nonapeptides, exceptions exist. For example, peptides eluted from HLA-Aw68 include peptides of 9, 10, 11 amino acids, which explains why only density of the first three and the last two amino acids but not the middle ones can be seen in the crystallographic structure (Guo et al, 1992).

Elution and sequencing of peptide pools from different alleles of the MHC class I have shown that each allele has its individual rules as to what peptides can be bound (Falk, et al, 1990, 1991; Rotzschke et al, 1990). In general, amino acids at only 2 or 3 positions out of the 9 are

conserved for a given allele. The discoveries of these allele-specific motifs obviously have significant practical applications, for example, in the area of vaccine design.

Although naturally processed peptides follow the specific motifs, the binding of MHC class I to peptides in vitro are much more flexible. In one report, even a pentapeptide can be presented by MHC class I (Reddehare, et al, 1989). This means that the chemical association by its own is not sufficient to determine a peptide being an MHC-restricted epitope. This may be another clue to suggest that something else is needed to charge peptides to MHC class I.

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