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Tumor rejection antigen gp96/grp94 is an ATPase: implications for protein folding and antigen presentation

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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. Furthermore, 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 an 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.

Key words: cancer immunity/chaperone/hsp90/MHC class I/peptide

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 are 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-related protein grp94 (Lee *et al.*, 1984; Mazzarella and Green, 1987; Srivastava *et al.*, 1987; 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 the basis of the observation that it is induced by accumulation of misfolded proteins in the endoplasmic reticulum (ER) (Kozutsumi *et al.*, 1988). In the absence of sequence differences in gp96 genes between tumors and normal tissues in spite of the tumor-specific antigenicity of gp96, we proposed (Srivastava and Heike, 1991; Srivastava and Maki, 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 gp96 was screened for the presence of motifs which are associated with ATP binding. The ATP-binding consensus sequences, types A and 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 downstream type B sequences (aa 231-241 and 303-313) were identified (Figure 1). Within the A type sequence, there are three amino acid residues between Gly217 and Gly221, 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

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	Type A							Type B						
Consensus	(G/A)X_4(G/A)(H/K/R)X_{0-1}(T/S/K/R/H)							(H/K/R)X_{5-8}ΦXΦ$_2$(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

Fig. 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), *E.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 acid. Lower case letters represent non-homologous residues. – indicates a gap. The highly conserved residues are boxed.

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 *Escherichia 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 [γ-^{32}P]8-azido ATP (Figure 2, lane 1). In parallel, ATP binding of hsp90 (as a positive control, lane 2) and an unrelated 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).

To determine whether gp96 interacts with ATP *in vivo*, EL-4 cells were labeled with ^{32}P and gp96 was immunoprecipitated from whole cell lysates of such cells. A phosphate-labeled gp96 band was observed (Figure 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 the case of hsp70, a number of bands other than the 70 kDa band were 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 whether the ^{32}P label in gp96 derives from associated ATP, or whether 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 (Figure 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.

Gp96 is an ATPase

Figure 4A shows the chromatographic profile of the last step of purification of Meth A gp96 on a Mono Q FPLC column.

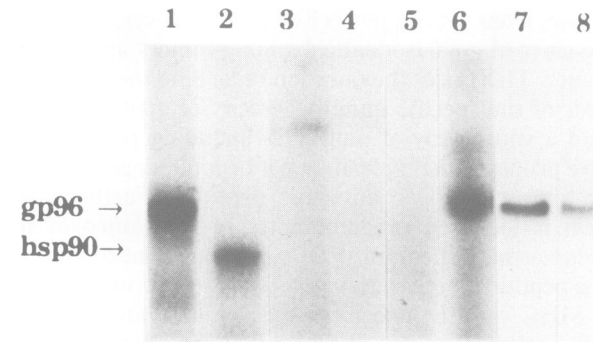

Fig. 2. Gp96 binds ATP *in vitro*. Binding of gp96 (lane 1), hsp90 (lane 2) or p96 (a Meth A-derived protein unrelated to gp96, lane 3) to [γ-^{32}P]8-azido was carried out as described in Materials and methods. Lanes 4–8: binding was carried out in the presence of 10 mM unlabeled 8-azido ATP (lane 4), ATP (lane 5), cyclic AMP (lane 6), GTP (lane 7) and CTP (lane 8).

Gp96 elutes within a broad range of salt concentration (0.45–0.6 M NaCl), instead of a sharp peak. However, the protein material in each peak comprises apparently homogeneous gp96 as judged by silver stained gels of these fractions (Figure 4B). The fractions were individually assayed for ATPase activity as described in Materials and methods. ATPase activity was clearly detected in the gp96 fractions (Figure 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 Figure 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 (Figure 5A, 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.

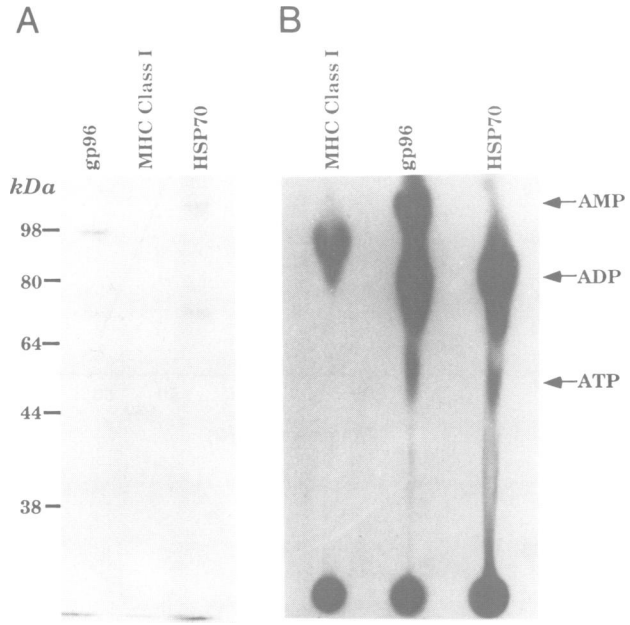

Fig. 3. Interaction of gp96 with adenosine nucleotide *in vivo*. (**A**) Immunoprecipitation of phosphate-labeled gp96 from [^{32}P]orthophosphate-labeled EL-4 cells. Immunoprecipitation was done with a polyclonal rabbit serum against MHC class I whole molecule (K270, obtained from Dr Per Petersen), 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). 1 μl of 100 μM ATP, ADP and AMP were spotted and localized with the aid of a short-wave UV lamp and the positions are indicated by arrows. The spot seen in the MHC class I lane on the TLC represents <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.

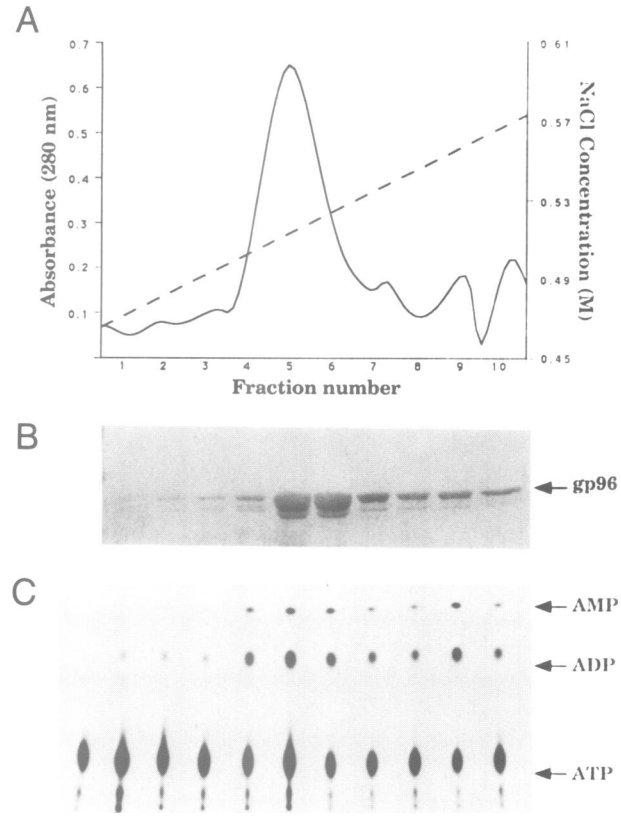

Fig. 4. ATPase activity co-purifies with gp96. (**A**) Chromatographic profile of gp96 on MonoQ FPLC. Proteins bound to concanavalin A−Sepharose column were dialyzed against 5 mM sodium phosphate buffer, pH 7.0, and applied to MonoQ FPLC column. Bound proteins were eluted with a linear 0−1 M NaCl gradient. Gp96 elutes at NaCl concentration between 0.45 and 0.6 M. (**B**) Silver stained SDS−polyacrylamide gel of MonoQ FPLC-purified fractions shown in (A). Fractions were applied to 10% SDS−PAGE and silver stained. Lanes 1−10 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 PEI thin layer chromatography and autoradiography. The positions of ATP, ADP and AMP are shown.

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 (Figure 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). Furthermore, the ATPase activity of gp96 was observed to be optimal at acidic pH (Figure 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 the 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 (Figure 6C). The other ER luminal HSP, 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 (Figure 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 K_m of 8 μM and the turnover rate is ~0.08 mol/min/mol. These characteristics suggest that ATP hydrolysis by gp96 plays a regulatory role (see Discussion).

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 (Carlino *et al.*, 1992; Gething and Sambrook, 1992). Moreover, ATP hydrolysis is stimulated by exogenous peptides (Flynn *et al.*, 1989, 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 8A, the

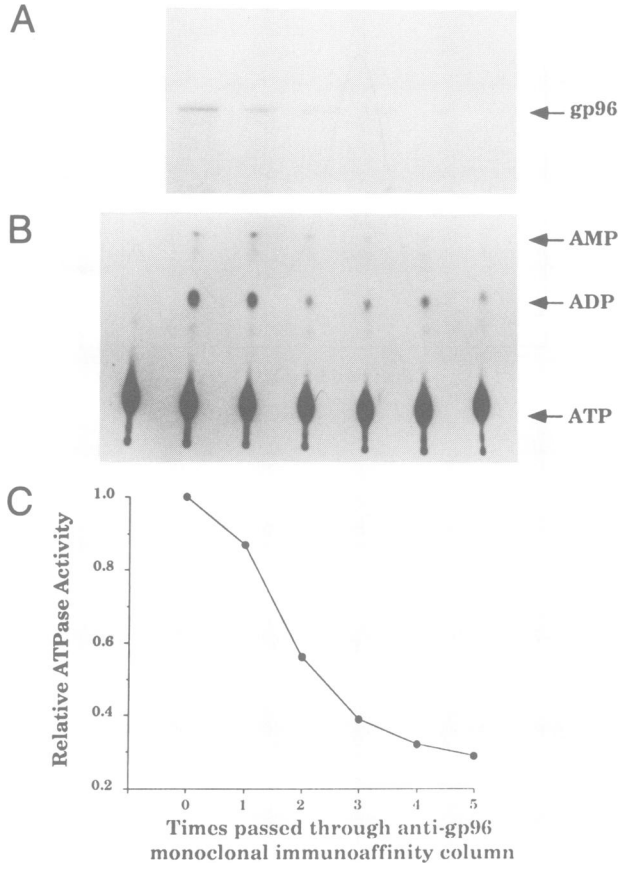

Fig. 5. ATPase activity resides in the gp96 molecule itself. **(A)** Depletion of gp96 using immunoaffinity column. Monoclonal antibody was coupled to protein G–Sepharose column. The same volume of original materials (~0.5 μg) and materials eluting from the column each time were applied to 10% SDS–PAGE 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 min. **(C)** Quantitative analysis of results in (B). 30 pmol ATP were hydrolyzed by 0.5 μg gp96 before the immunodepletion and the relative ATPase activity was defined as 1.

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. An 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 ~1.5-fold by addition of peptides (Figure 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.

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

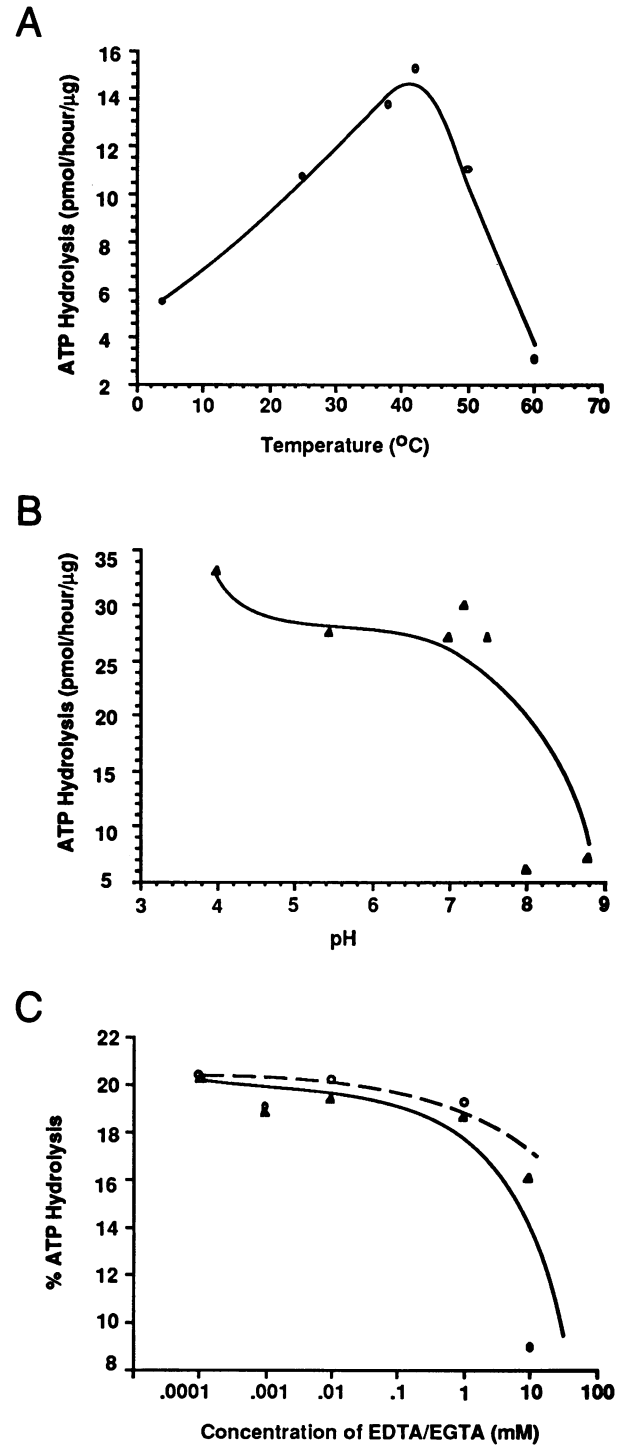

Fig. 6. Effect of temperature, pH and divalent cations on the ATPase activity associated with gp96. **(A)** Effect of temperature on the rate of ATP hydrolysis by gp96. **(B)** Optimization of pH. 570 ng purified gp96 were assayed for ATPase activity in the presence of 20 mM NaCl, 2 mM $MgCl_2$ and 20 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).

or so-called 'folding intermediates' or 'molten globules' (for reviews, see Ang *et al.*, 1991; Gething and Sambrook, 1992). Thus, groEL does not recognize either completely folded or totally unfolded polypeptides, nor does it bind

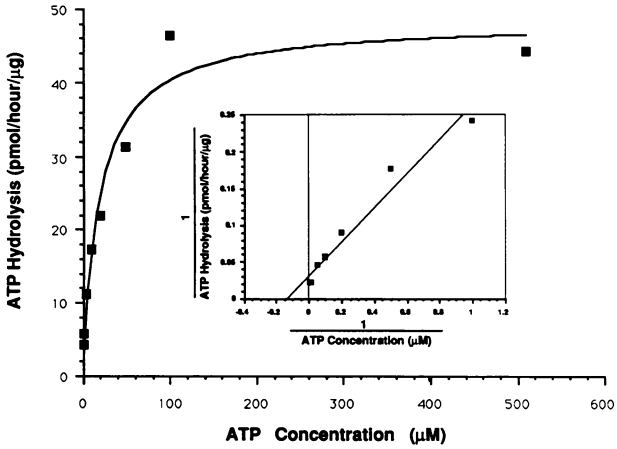

Fig. 7. Kinetic study of gp96 ATPase activity. Substrate saturation experiment was carried out by incubating purified gp96 (0.862 μg per reaction) with increasing concentrations of [α-^{32}P]ATP in 20 mM HEPES buffer, pH 7.2, containing 20 mM NaCl and 2 mM $MgCl_2$ at 37°C for 1 h. Background values in the absence of gp96 have been subtracted. Double reciprocal plot of the same data is shown in inset. The K_m calculated from this experiment is 8 μM.

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 (Figure 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 Da or less), as described in Materials and 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 (Figure 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 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 1000 Da. 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 that include elution on a salt gradient on which gp96 elutes at ~0.5 M NaCl. The final gp96 preparation may be used immediately

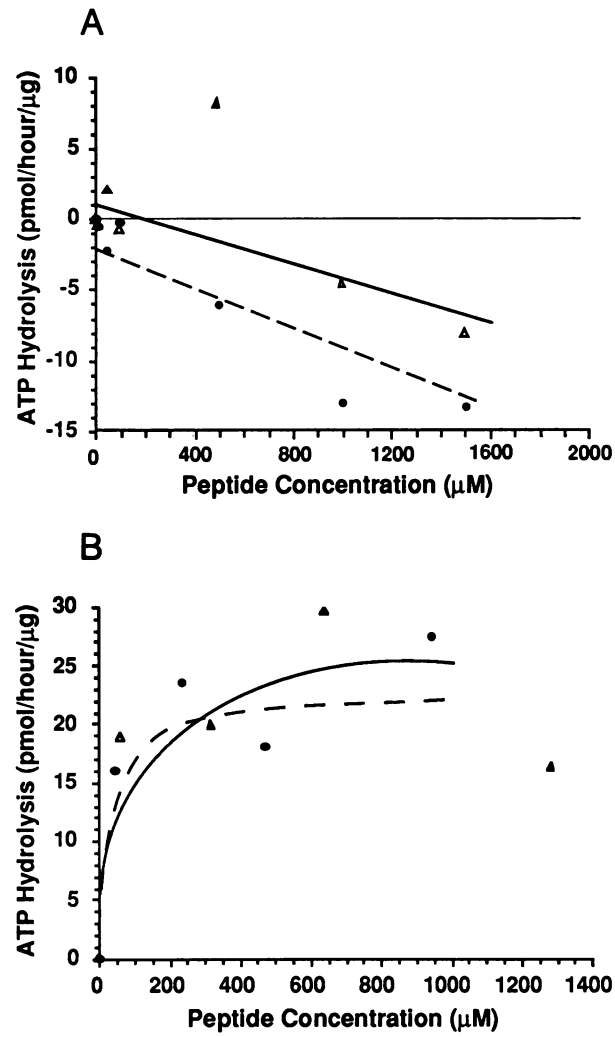

Fig. 8. Modulation of ATPase activity of gp96 and hsp70 by peptides. (**A**) Peptides do not stimulate ATPase activity of gp96. 1 μg of purified gp96 was assayed for ATPase activity in the presence of increasing concentrations of either peptide A (△—△) or peptide B (●-----●) at 37°C for 1 h. Both peptides A and B are 15mers and are derived from vesicular stomatitis virus glycoprotein (peptide A, KRQIYTDLEMNRLGK; peptide B, LSSLFRPKRRPIYKS). Peptide-independent ATP hydrolysis of 50 pmol/h/μg was subtracted. (**B**) Peptides stimulate ATPase activity of hsp70. The same peptide preparation used in (A) was used. Peptide-independent ATP hydrolysis of 85 pmol/h/μg has been subtracted.

for acid extraction or it may be stored in this high salt buffer for several hours in the presence of high concentrations of protease inhibitors (2 mM PMSF, 10 μM leupeptin) at 4°C, before acid extraction. In either case, we routinely centrifuged the purified preparations in the high salt buffer through a centricon-10 immediately preceding acid extraction and did 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).

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

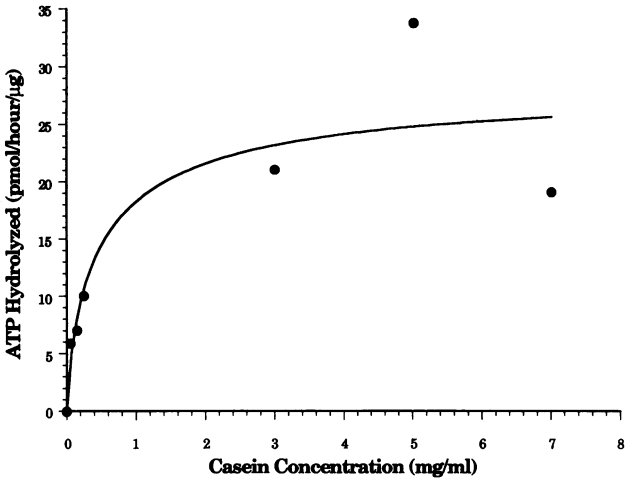

Fig. 9. Casein stimulates ATPase activity of gp96. ATPase activity of gp96 was assayed in the presence of increasing concentrations of casein. ATPase activity with gp96 alone as well as the minor background ATP hydrolysis associated with casein itself are subtracted from each point.

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 an Mg^{2+}-dependent ATPase. The deduced amino acid sequence of gp96 contains ATP-binding motifs and gp96 binds ATP *in vitro* and *in vivo* (Figures 2 and 3). The K_m of the ATPase activity was calculated as 8 μM and the maximum velocity as 50 pmol/h/μg (Figure 7). This corresponds to a turnover rate of 0.08 mol/min/mol. 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.coli* hsp70 dnaK has a K_m value of $\sim$20 μM and a turnover rate of 0.2 mol/min/mol (Liberek *et al.*, 1991). Grp78, an hsp70 member of the ER, has a K_m of $\sim$0.1 μM and a turnover rate of $\sim$0.34 mol/min/mol (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 mol/min/mol (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. Furthermore, 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

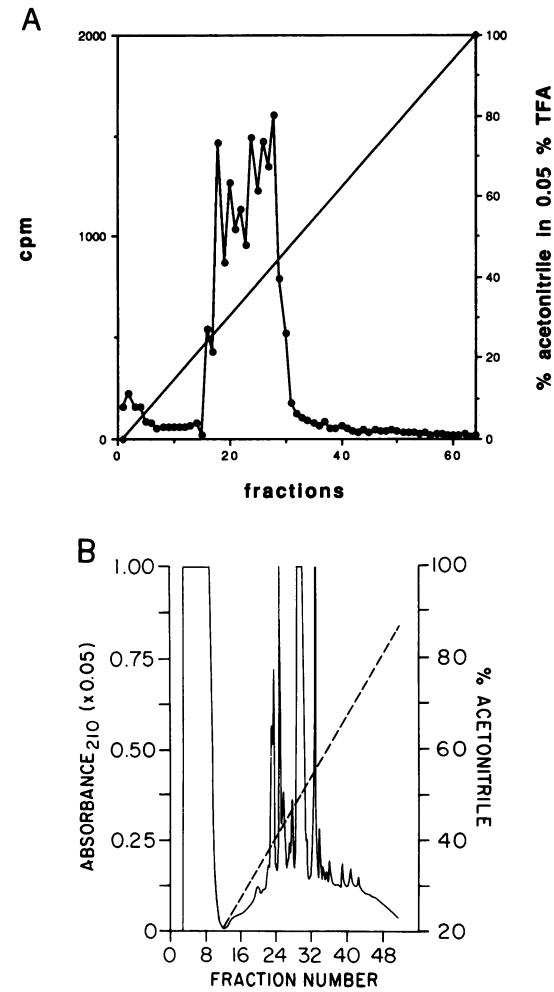

Fig. 10. Gp96 is associated with heterogeneous peptides. Purified gp96 was extracted with 10% acetic acid. Peptides eluted from gp96 were separated through centricon-10 and separated on a C_{18} HPLC column, as described in Materials and methods. (**A**) Peptides eluted from gp96 derived from 1×10^9 Meth A cells metabolically labeled with [^{35}S]methionine. (**B**) Peptides isolated from macaque liver gp96.

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. Similar to grp78, grp94 has been shown to be induced by accumulation of malfolded proteins in the ER (Kozutsumi *et al.*, 1988) 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 (Z.Li and P.K.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, 1991). 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, 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; 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 (Figures 8 and 9) may suggest an additional, unique role for gp96. Gp96 is among the most recently evolved HSPs. It is not found in yeasts 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 carboxy-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, which 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 (Z.Li and P.K.Srivastava, in preparation), we suggest that one of the specific functions of gp96 involves charging of MHC class I molecules with peptides.

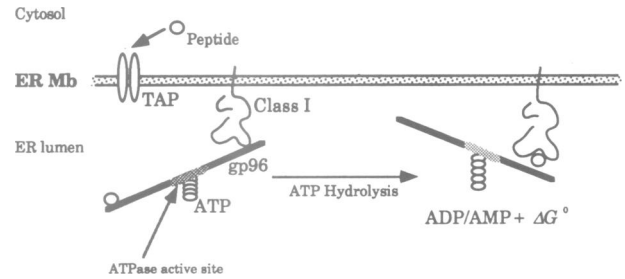

Fig. 11. A model for the role of gp96 in MHC class I peptide assembly. It is postulated that the interaction of MHC class I and gp96 stimulates the ATPase activity of gp96. The free energy (ΔG^0) released due to ATP hydrolysis induces a conformational change in gp96, leading to transfer of peptides from gp96 to MHC class I. TAP denotes transporter-associated proteins.

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

We demonstrate that gp96 is associated with a wide array of peptides (Figure 10). In the context of our observation of co-immunoprecipitation of gp96 and MHC class I from metabolically labeled cells (Z.Li and P.K.Srivastava, in preparation) and in light of the ATPase activity of gp96, we propose (Figure 11) that (i) gp96 acts as a repository of the peptides transported into the lumen of the ER, (ii) interaction of gp96 with MHC class I stimulates its ATPase activity, resulting in hydrolysis of gp96-bound ATP, (iii) 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 (Alexander *et al.*, 1989, 1990; Rothman, 1989; Townsend *et al.*, 1990; Levy *et al.*, 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; Wada *et al.*, 1991; Galvin *et al.*, 1992; Hochstenbach *et al.*, 1992). It is a membrane-bound (as opposed to luminal) protein and is associated with partially 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 (Sibille *et al.*, 1990; Srivastava, 1990; Szikora *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.

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 an 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 [γ-^{32}P]8-azido ATP in a final volume of 100 μl was irradiated with a short wavelength (254 nM) UV source from a distance of 8–10 cm for 4 min (Hobson *et al.*, 1984). Samples were then 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.* (1989, 1991). Typically, 1 μg of purified gp96 or hsp70 was incubated with 20 μM [α-^{32}P]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 h. 1 μl of the reaction mixture was then spotted onto a polyethyleneimine (PEI) cellulose plate. Thin layer chromatography was performed against 1:1 ratio of 1 M LiCl and 1 M HCOOH. The plate was then dried, exposed to film and corresponding radioactive spots were excised and counted. ATPase activity was determined from the amount of [^{32}P]ADP and [^{32}P]AMP generated from [α-^{32}P]ATP, i.e. the percentage of ATP hydrolyzed calculated as [ADP + AMP]/[ATP + AMP + ADP] × 100%. Background ATP hydrolysis lacking purified gp96 or hsp70 was subtracted.

Phosphate labeling and immunoprecipitation

Labeling cells with ^{32}P was done according to Downward *et al.* (1990). Immunoprecipitation was carried out using the 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 blotting, silver staining and immunoaffinity protein G columns

Western blotting was done according to the 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 a centricon-10 spin column (MW cut off 10 000 Da, 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 a C_{18} column and acetonitrile gradient with a flow rate of 1 ml/min (buffer A, 0.05% TFA; buffer B, 100% acetonitrile, the gradient is linear from 0 to 100% buffer B). 1 ml fractions were collected.

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