An Endoplasmic Reticulum Protein Implicated in Chaperoning Peptides to Major Histocompatibility of Class I Is an Aminopeptidase*

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CD8+ T lymphocytes recognize antigens as peptides bound to MHC I molecules. The peptides are generated by degradation of cellular proteins in the cytosol and are transported into the endoplasmic reticulum (ER) through transporter associated with antigen processing (TAP) molecules (1). A number of proteins, such as calnexin and tapasin (2, 3), promote the assembly of the trimolecular MHC I-β2-microglobulin-peptide complex in the ER. Stable binding of a peptide to a given MHC I allele requires the peptide to be 8–9 amino acid residues long and to possess a sequence motif characteristic of that allele (4, 5). Since the peptides are generated in the cytosol in the absence of guidance by the MHC I molecules, the mechanism whereby peptides with an appropriate MHC I binding length and sequence are generated is unclear. The proteasomes generate peptides of ∼8–9 amino acid residues, among longer peptides. Such peptides also generally contain the correct hydrophobic carboxyl terminus, which is required for binding to most MHC I alleles (6–8). The amino terminus of the peptides is more variable. It has been shown previously that TAP-transported peptides undergo further editing in the early secretory pathway by putative aminopeptidases, which trim the peptides to their final size (6–12). We report here the identification of an aminopeptidase activity of an ER luminal chaperone, which (i) is the most abundant component of the ER lumen (13–15), (ii) binds peptides transported into the ER through TAP and other mechanisms (16), and (iii) is an ATPase (17). The ability of this aminopeptidase to trim long peptides for optimal MHC I binding is demonstrated functionally.

MATERIALS AND METHODS

Protein Purification—gp96, hsp70, and hsp90 have been purified as described previously (18). Recombinant mouse gp96, WTgp96 and Ala655 gp96, were obtained by expression of murine gp96 cDNA in JM109F Escherichia coli. Bacteria were lysed in binding buffer (20 mM phosphate, 500 mM NaCl, pH 7.8, 10 mMimidazole, 1 mM PMSF) in the presence of lysozyme. The lysate was saturated with 60% ammonium sulfate, and the precipitated proteins were applied to and eluted from Probond™ metal-binding buffer (Invitrogen). The gp96 preparations (mouse and recombinant) were repurified by HPLC on POROS-HQ column and eluted by a linear gradient from 0 to 1 M NaCl. The purified fractions were checked for purity by SDS-PAGE and immunoblotting. The immunoaffinity-purified gp96 was a generous gift of Hansjoerg Schild. gp96 was purified using a recombinant single-chain Fv fragment (scFv antibody) generated from a semi-synthetic phage library using mouse native gp96 molecules as described previously (19). All proteins were quantified using the Bradford assay, and bovine serum albumin was used as the standard (Bio-Rad).

Two-dimensional Gel Electrophoresis—gp96 in 20 μl of isoelectric focusing buffer (9.5%urea, 2% CHAPS, 100 mM dithiothreitol, and 2% carrier ampholines: 1% pH 5–7, 0.6% pH 3–10 (Amersham Pharmacia Biotech, Uppsala, Sweden) was loaded into a capillary tube containing 2.9% acrylamide (Bio-Rad), 2% CHAPS, 9.5 μm urea, and 2% Ampholines, pH 5–7–10. The proteins were focused for 3200 V as described previously (20). After extrusion, gels were loaded onto a 10% SDS-polyacrylamide slab and electrophoresed at 200 V.

Protease Assays—The proteolytic activity of gp96 was tested using various endo- and exopeptidase substrates (Novabiochem, Laufelfingen, Switzerland) composed of a mono or a poly amino acid peptide covalently bound to the chromophore p-nitroanilide (pNA). Substrates were incubated with proteins at 37 °C in sterile buffer (5 mM phosphate, 0.7 μM NaCl, 0.02% NaN3 at pH 7.0). Hydrolysis of the polypeptide chain was quantified by recording the optical density at 405 nm.

RESULTS

Identification of an Aminopeptidase Activity in gp96 Preparations—Homogeneous preparations of gp96 were previously observed to degrade spontaneously into smaller sized polypeptides at 4 °C (18, 22), thus generating a ladder-like pattern of bands on SDS-polyacrylamide gels (Fig. 1A), suggesting that either gp96 had a proteolytic activity or that the preparations contained a contaminant. The gradual appearance of incrementally smaller sized bands suggested that the activity was not an endopeptidase, but an exopeptidase.

gp96 purified to homogeneity, as judged by a single band on
SDS-PAGE after silver staining, was tested for aminopeptidase and endopeptidase activities using synthetic substrates. This led to identification of an aminopeptidase activity associated with gp96 preparation (Fig. 1B). The purified preparation was tested for cathepsin G, chymotrypsin, thermolysin, elastase, and subtilisin activities using their specific substrates; no such activities were detected (Fig. 1B). The aminopeptidase activity was abrogated by inhibitors of aminopeptidases such as bestatin and amastatin but not by cystatin, which does not inhibit aminopeptidases (Fig. 1C). PMSF, a serine protease inhibitor, was observed to significantly inhibit the aminopeptidase activity of gp96, albeit at relatively higher concentrations of the inhibitor. Interestingly, another aminopeptidase, a rat aminotripeptidase, has been shown previously to be inhibited by bestatin as well as by PMSF (23). The aminopeptidase activity of gp96 was observed to be calcium-dependent and optimal at 40 °C and a pH of 6.5 (data not shown).

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FIG. 1. gp96 is an aminopeptidase. A, purified gp96 preparation degrades spontaneously into a broad range of smaller polypeptides. gp96 purified from Meth A cells, as described previously (22), and further repurified on anion exchange HPLC column (POROS-HQ) as a homogeneous protein, was immediately stored at −20 or at 4 °C for 3 weeks and analyzed by SDS-PAGE. B, the proteolytic activity of gp96 was tested using various endo- and exopeptidase substrates. Substrates were incubated for 16 h at 37 °C in sterile buffer with 2 μg of gp96. Hydrolysis of the polypeptide chain was quantified by recording the optical density at 405 nm. The results shown here are under linear condition of assay for each substrate. C, the inhibition of the aminopeptidase activity of the gp96 preparation was tested in the presence of protease inhibitors. Hydrolysis of 2 mM concentration of the aminopeptidase substrate Leu-pNA by 2 μg of gp96 (under the same condition as in B) was inhibited in the presence of bestatin and amastatin (aminopeptidase inhibitors), PMSF (serine protease inhibitor) or cystatin (cysteine protease inhibitor). Other protease inhibitors, including HgCl2 (aminopeptidase inhibitor), apoprotein (serine protease inhibitor), leupeptin (serine and cysteine protease inhibitor, cathepsin B inhibitor), antipain (trypsin-like inhibitor), dipeptidyl A (aminopeptidase inhibitor), E64 (cysteine protease inhibitor), and antipain (papain, trypsin, cathepsin A and B inhibitors) had no effect on the aminopeptidase activity associated with the gp96 sample (data not shown).
ent and different method. gp96 was purified by immunoaffinity chromatography using single chain antibody to gp96 (19). The antibody is a single chain Fv fragment (scFv antibody) generated from a semi-synthetic phage library using mouse native gp96 molecules. The scFv antibody clone H11B was immobilized on CNBr-Sepharose 4B, and hypotonic cell lysates were passed over it. Elution of gp96 was performed using phosphate-buffered saline containing 1.3M NaCl. Immunoaffinity-purified and conventionally purified gp96 were compared for their specific aminopeptidase activities: no differences were observed (Fig. 2).

FIG. 2. The aminopeptidase activity of gp96 is inherent in the gp96 polypeptide chain. A, gp96 purified by the classical biochemical method (18) and by immunoaffinity chromatography using single chain recombinant antibody (as described in Ref. 19 and under "Materials and Methods") were incubated with the aminopeptidase substrate Leu-pNA as described in the legend to Fig. 1. B, co-localization of gp96 and the aminopeptidase activity on a two-dimensional polyacrylamide gel. Three two-dimensional gels were run simultaneously under the same conditions (as described in Ref. 20), with the identical gp96 sample. One gel was silver-stained, a second gel was immunoblotted with a monoclonal antibody specific for gp96, and the third gel was cut into 10 acrylamide pieces (1 mm x 1.5 mm x 1.5 mm). C, each piece was used to elute any proteins, as described under "Material and Methods," and the eluate was assayed for its ability to degrade the aminopeptidase substrate Leu-pNA.

FIG. 3. The recombinant gp96 possesses an aminopeptidase activity. A, purified mouse gp96 and recombinant gp96 (WT96) were analyzed by silver-stained SDS-PAGE (left panel) and immunoblot (right panel) using anti-gp96 9G10 monoclonal antibody. The slight increase of molecular weight of WT96 is due to its additional amino-terminal His-tag. B, mouse gp96 and WT96 were tested as in Fig. 1B except that the buffer was supplemented with 0.2% n-octyl-β-D-glucopyranoside.

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were confirmed to be gp96 by immunoblotting of a second gel with a monoclonal antibody (specific for gp96 (Fig. 2B). (The monoclonal antibody 9G10 was not raised nor selected using purified gp96 preparations but by independent methods, see Ref. 24.) A third gel was dissected into slices in form of a grid; proteins were eluted from each slice and were analyzed by SDS-PAGE and immunoblotting and for aminopeptidase activity following renaturation of gp96. As in case of a number of other enzymes (25), the aminopeptidase activity of gp96 could be partially renatured following electrophoresis in SDS-PAGE. The results in Fig. 2B show that: (i) all slices that contain gp96 possess an aminopeptidase activity, (ii) an aminopeptidase ac-

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**Fig. 4.** The aminopeptidase activity of gp96 is compromised by a point mutation. A, purified wild type gp96 (WTr96) and recombinant gp96 mutated on position 655 (Ala655 r96) were stored at −20 °C or +37 °C for 72 h and analyzed by silver-stained SDS-PAGE (left panel) and immunoblot (right panel) using anti-gp96 9G10 monoclonal antibody. B, the aminopeptidase activity of WTr96 and Ala655 r96 was tested as in Fig. 1B except that the buffer was supplemented with 0.2% n-octyl-β-D-glucopyranoside. C, the aminopeptidase activity of gp96 is not shared by other HSPs. The HSPs gp96, hsp70, and hsp90 (purified as described in Ref. 18) and phosphorylase b were tested for their ability to degrade Leu-pNA as described in the legend to Fig. 1B.
Activity is not detected in any slices other than those that contain gp96, and (iii) gp96 is not detected in any slices other than those that contain an aminopeptidase activity (Fig. 2C).

The aminopeptidase activity of recombinant gp96 was tested. Murine gp96 was expressed in E. coli and purified to homogeneity. The identity of r-gp96 as gp96 was confirmed by immunoblotting with the 9G10 anti-gp96 antibody (Fig. 3A). The r-gp96 and murine gp96 were assayed for aminopeptidase activity in titrated quantities; the two preparations were observed to have identical specific aminopeptidase activities (Fig. 3B), thus indicating that the aminopeptidase activity of murine gp96 was not a contaminant but an inherent component of the gp96 polypeptide.

To test this point further, we sought to introduce mutations in gp96 that might affect the aminopeptidase activity of gp96. The gp96 sequence was scanned for homology with known aminopeptidases and aminopeptidase active sites for the presence of a common motif. No such motif was detected. However, as the aminopeptidase activity of gp96 showed sensitivity to PMSF, which is a serine protease inhibitor, a serine protease motif GXSG (26–28) was sought. An imperfect motif, GWSG, was detected at positions 653–656. The murine gp96 gene cloned in E. coli was mutated such that the serine at position 655 was altered to an alanine (Ala655 gp96). The Ala655 r96 mutant was observed to be severely compromised in aminopeptidase activity as compared with the wild type r96 (Fig. 4, A and B). Further evidence of differences in activity of the wild type and Ala655 preparations was observed in an in situ assay of aminopeptidase activity (Fig. 4B). The wild type and the Ala655 gp96 preparations were stored frozen at –20 °C or incubated for 72 h at 37 °C and were analyzed by SDS-PAGE and immunoblotting of the gels with anti-gp96 monoclonal antibody 9G10. Both preparations, stored at either temperature, showed apparently homogeneous bands of 96 kDa on SDS-PAGE. Immunoblotting of the gels showed that both preparations were stable at –20 °C. However, the wild type preparation had begun to degrade at 37 °C, creating the ladder-like patterns seen in Fig. 1, while the Ala655 mutant preparation was still stable at 37 °C. These results are consistent with the abrogation of the aminopeptidase activity in the Ala655 mutant. These data do not imply that the Ala655, or the GWSG site, constitutes the active site of gp96; rather they simply show that a mutation in the primary structure of gp96 can affect its activity profoundly, thus offering extremely strong evidence that the activity is not a contaminant.

**gp96 Is Unique among HSPs Tested with Respect to Its Aminopeptidase Activity**—ER-resident gp96 shares significant homology with the cytosolic hsp90. Neither hsp90 and the unrelated cytosolic HSP, hsp70, nor a control protein phosphorylase b, showed any detectable aminopeptidase activity at all at any of the titrated quantities (10–50 μg) tested (Fig. 4C).

**Trimming Activity of gp96 Aminopeptidase on an NH2-Terminally Extended MHC I Epitope**—The possibility that the aminopeptidase activity of gp96 molecules can trim peptides longer than 8 or 9 amino acids to MHC I epitopes was tested directly. The ability of precise 8-mer epitopes to sensitize target cells for recognition and lysis by cognate CTLs, and the inability of longer precursors to do so, was used as an assay. A K°-restricted CTL clone against the vesicular stomatitis virus nucleocapsid protein epitope NH2-RGYVYQGL-COOH (VSV8) was used (Fig. 5). While sensitization of EL4 cells with VSV8 leads to antigen-specific lysis by VSV8-specific CTLs, no significant recognition of an amino terminus-extended 19-mer peptide VSV19 (NH2-AATYQRTRALVRGYVYQGL-COOH) was observed (Fig. 5A). However, treatment of VSV19 with increasing quantities of gp96, but not with an unrelated 93-kDa protein, phosphorylase b, leads to an increasing recognition of sensitized EL4 cells, such that at the maximum concentration tested, the VSV8 and gp96-treated VSV19 show an identical ability to sensitize EL4 cells for recognition by the cognate CTLs (Fig. 5B). The cytotoxicity is not due to the presence of gp96 in the assay as pulsing of EL4 cells with low molecular mass fractions (less than 10 kDa) of gp96-digested VSV19 confers equal susceptibility to CTL-mediated killing (Fig. 5C).

**DISCUSSION**

Our experiments show that the ER-resident gp96 is an aminopeptidase and that it can trim NH2-terminally extended peptides. These results are significant because of the now long held belief that the ER must harbor an aminopeptidase activity that can act on TAP-transported antigentic peptides, which have precise MHC I-binding carboxyl termini but variable and extended amino termini (6–12). gp96 fulfills the expectations for a candidate for this activity. The present studies show that
gp96 is an aminopeptidase that can trim N-terminally extended precursor peptide for optimal MHC I binding and CTL recognition. Previous studies have indicated that gp96 is a major component of the ER lumen (13–15), that it associates with a wide array of antigenic peptides generated in the cytosol or in the secretory pathway (16, 17), and that it binds with peptides transported into the ER through both TAP-dependent (29) and TAP-independent mechanisms (30). Collectively, these considerations make a compelling case for the proposition that gp96 is responsible for further processing of antigenic peptides in the ER, a phenomenon that is well described (6–12) but remains poorly defined.

Several points are of note in this regard. While our studies show that gp96 can trim VS/V19 to VS/V8, they do not show nor imply that the aminopeptidase activity of gp96 stops at the precise amino terminus necessary for binding to MHC I. That specificity must come from the protective action of the MHC I molecule itself. Second, we must point toward the relatively low aminopeptidase and ATPase enzymatic activities of gp96 in assays in vitro. The ATPase of gp96 has been previously estimated to be $2.5 \times 10^7$ times lower than the corresponding activity of adenylate kinase (17); the aminopeptidase activity of gp96 is approximately $5 \times 10^6$ times lower than the corresponding activity of the aminopeptidase-M (data not shown). It is our belief that the two enzymatic activities are enhanced or otherwise modulated in vivo by presently unidentified co-factors. Third, gp96 is an aminopeptidase while the closely related hsp90, the cytosolic homologue of gp96, is not, indicating that this activity is not a characteristic of chaperones in general. Hsp90 genes are present and expressed in all forms of life, this activity is not a characteristic of chaperones in general. Hsp90 genes are present and expressed in all forms of life, although clearly derived from them (31, 33), Hsp90, the cytosolic homologue of gp96, is not, indicating that hsp90 molecules (17). These two activities appear to have been incorporated into hsp90 genes, along with the addition of an amino-terminal signal peptide and a carboxyl-terminal ER retention sequence, possibly for an ER-specific function. Finally, the observation that gp96 harbors ATPase and aminopeptidase activities is surprising, but it is not inconsistent with the multiple enzymatic activities of other HSPs. Protein disulfide isomerase, another chaperone of the ER lumen, is an ATPase and a disulfide isomerase (34). Lon, a mitochondrial chaperone, is an ATPase as well as a serine protease (26).

Our results do not permit us to make the case that the aminopeptidase activity of gp96 is essential for antigen presentation by MHC I molecules. The classical genetic tools, which are used so elegantly to make such determinations, are unavailable to us because of the versatile role of HSPs in processes other than antigen presentation. Thus, gp96 knockout mice are embryonic lethal.2 However, our data firmly place gp96 molecules at the scene of the crime and in possession of the tools of the crime.

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