GRP94-associated Enzymatic Activities

RESOLUTION BY CHROMATOGRAPHIC FRACTIONATION*

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GRP94 (gp96), which performs established functions as a molecular chaperone and immune system modulator, has been reported to display a number of intrinsic enzymatic activities, including ATP hydrolysis, protein phosphorlation, and aminopeptidase. In observing that GRP94 co-purified with bacterial β-galactosidase through multiple chromatographic steps, we have examined the hypothesis that the reported enzymatic activities of GRP94 may reflect co-purification of contaminant enzymes, rather than intrinsic catalytic functions. In subjecting GRP94 to increasingly stringent chromatographic purification, we report that a GRP94 carboxyl-terminal directed protein kinase activity could be separated from GRP94 by heparin affinity chromatography. Analysis of the kinase substrate specificity indicates that this kinase is distinct from casein kinase II, which is known to co-purify with GRP94. Electrophoretically pure GRP94 displayed low, but significant levels of aminopeptidase activity. Further purification of GRP94 by anion exchange and heparin affinity chromatography yielded resolution of GRP94 from the aminopeptidase activity. Furthermore, exhaustive trypsinolysis of GRP94 preparations displaying aminopeptidase activity yielded complete proteolysis of GRP94 but did not affect aminopeptidase activity. These results are discussed with respect to current models for GRP94 function and the role of such co-purifying (poly)peptides in the generation of GRP94-dependent cellular immune responses.

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1 The abbreviations used are: ER, endoplasmic reticulum; CKII, casein kinase II; APC, antigen-presenting cell; rVV, recombinant vaccinia virus; ConA, concanavalin A; Ala-pNA, alanine-p-nitroanilide; MHC, major histocompatibility complex; FPLC, fast protein liquid chromatography; CPRG, chlorophenol red-β-D-galactosidase; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]1-propanesulfonic acid.

2 R. C. Reed and C. V. Nicchitta, unpublished observations.
contaminating polypeptides. Thus, when the apparent specific activity of an enzyme is low, it is difficult to distinguish between activity intrinsic to the protein and that which is derived from contaminants. In fact, such confusion has been previously chronicled with respect to heparanase, which has been proposed to be highly homologous with, or identical to, GRP94 (20, 21). Here, we report evidence that the self-directed kinase and aminopeptidase activities reported for GRP94 are caused by co-purifying proteins, rather than GRP94 itself.

EXPERIMENTAL PROCEDURES

Materials—Geldanamycin and radicicol were kindly provided by Dr. Len Neckers (National Cancer Institute, National Institutes of Health, Rockville, MD). Chlorophenol red-β-galactopyranoside (CPRG) was obtained from Roche (Basel, Switzerland). [γ-32P]ATP was purchased from PerkinElmer Life Sciences. [32P]Orthophosphate was purchased from Amersham Biosciences. DU-120 is a rabbit polyclonal antibody directed against a domain in the GRP94 NH2 terminus and was prepared by contract service with Cocalico Biologicals (Reamstown, PA). All other reagents were purchased from Sigma-Aldrich.

Expression and Purification of GRP94, C[187], and N(22–337)—GRP94 was purified from the livers of C57/B16 mice or from porcine pancreas by either of two methods (18, 19). For the latter method (19), the following changes were made: all chromatographic steps were performed on an FPLC system (Amersham Biosciences), and K-HEPES-buffered saline was substituted for phosphate-buffered saline during ConA lectin affinity chromatography. Additionally, ResourceQ anion exchange resin (Amersham Biosciences) was substituted for DEAE anion exchange resin and elution was performed with a linear gradient of 50–750 mM NaCl in 10 mM Tris-Cl (pH 7.8). For the former method (18) began with the generation of rough ER microsomes, as described in Walter and Blobel (22), and was otherwise identical to that of Wearsch and Nicchitta (18).

Canine GRP94 cDNA was used as the template for all PCR reactions. To prepare C[187], which represents the carboxyl-terminal 187 amino acids of mouse GRP94, the sense primer (5′-GGATCCTCAATTCATAAGCTCCCAATCCCA-3′) and a 3′ antisense primer (5′-CGTTCGACCCCTCTCCACACAGGA-3′) were used to obtain a PCR product corresponding to the 3′ 558 bp (bp 1565–2151) of the GRP94 coding region, flanked by 5′ BamHI and 3′ PstI restriction sites. The PCR product was digested with BamHI/PstI and subsequently ligated into BamHI/PstI-digested pSET-B (Invitrogen). For N(22–337), which represents the aminoterminal cleavage comprising amino acids 22–337, a 5′ antisense primer (5′-GGATCCCATATGGACGATGAAGTCGATGTG-3′) and a 3′ antisense primer (5′-GGATCCTCAATTCATAAGCTCCCAATCCCA-3′) were used to obtain a PCR product corresponding to bp 64 to 1004 of the GRP94 coding region, flanked by 5′ NdeI and 3′ BamHI restriction sites. The PCR product was digested with NdeI/BamHI and ligated into the NdeI/BamHI-digested pEUX vector (gift of Dr. Daniel Lewin, Duke University Medical Center, Durham, NC).

All protein purifications were performed at 4 °C. Recombinant C[187] protein was expressed in Echerichia coli strain BL21 and purified using Ni2+-nitrilotriacetic acid beads (Qiagen Sciences, Germantown, MD) in 8 M urea, according to the manufacturer’s protocol. After elution, the protein was dialyzed overnight against two 500-fold volume changes of buffer containing 50 mM Tris-Cl (pH 7.5), 500 mM NaCl, 10% (v/v) glycerol, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 mM β-mercaptoethanol. The protein was subjected to additional purification on a Sephacryl S-200 gel filtration column, in buffer containing 25 mM K-HEPES (pH 7.4), 20 mM NaCl, 100 mM KCl, 2 mM MgCl2, and 0.1 mM CaCl2. It was then concentrated by centrifugal ultrafiltration and frozen in aliquots. Recombinant N(22–337) was expressed in E. coli strain BL21 and purified using a GSTrap affinity column (Amersham Biosciences) according to the manufacturer’s protocol. Removal of glutathione S-transferase was accomplished by thrombin digestion, and further purification was performed using Superdex 75 gel filtration chromatography (Amersham Biosciences). The purified N(22–337) protein was then concentrated by centrifugal ultrafiltration and frozen in aliquots.

**Determination of Enzyme Activities—β-Galactosidase activity was assayed using CPRG chromogenic substrate. 200 μl reactions containing 5 μM CPRG, 50 mM potassium phosphate buffer (pH 7.8), and 1 mM MgCl2 were incubated with samples or dilutions of purified β-galactosidase at 37 °C until color was visible (generally about 20 min) and the A540 was determined. All β-galactosidase activities were corrected for background substrate hydrolysis (reaction containing no added sample protein).

**RESULTS**

**GRP94 Kinase activity was assayed in 25-μl reactions containing 50 mM K-HEPES (pH 7.4), 10 mM MgCl2, and 0.15 μM [γ-32P]ATP (2.67 × 106 cpm/mmol). Where indicated, GRP94 was present at 2.4 μg/assay, whereas C[187] was present at 0.75 μg/assay. Incubations were performed at 37 °C for 60 min and stopped by addition of 3 μl of 100 mM H2O. Following incubation on ice and centrifugation, the pellet was re-suspended in SDS-PAGE sample buffer and analyzed by SDS-PAGE, followed by phosphorimager analysis on a Fuji MacBas100 phosphorimager (Fuji Medical Instruments, Stamford, CT). Quantification was performed using MacBas version 2.0 software (Fuji Medical Instruments).

β-Galactosidase activity was determined by the method of Menoret et al. (16), using 20 μM alanyl-p-nitroanilide (Ala-pNA) (Sigma) as a substrate. Incubations were performed at 37 °C for 16 h in the dark. All values were corrected for background hydrolysis in samples lacking added protein.

**Chromatographic Fractionation of Enzyme Activities—** Purification of GRP94 from endoplasmic reticulum-derived microsomes was performed by the method of Wearsch and Nicchitta (18). All chromatographic fractionations were performed on an FPLC system at 4 °C. For ResourceQ chromatography, a 10-ml ResourceQ column was eluted with a linear 70-ml, 50–750 mM NaCl gradient in 10 mM Tris-Cl (pH 7.8). Fractions of 2 ml were collected. For heparin chromatography, a 5-ml heparin HiTrap column (Amersham Biosciences) was eluted using a linear 35-ml, 0–2 M NaCl gradient in 20 mM K-HEPES (pH 7.4). Fractions of 0.5 ml were collected.

For ConA lectin affinity chromatography, protein was loaded on a 3-ml ConA-agarose column (Sigma) and washed extensively in a column buffer containing 30 mM K-HEPES (pH 7.4), 150 mM NaCl, 1 mM CaCl2, and 1 mM MgCl2. To elute ConA-bound proteins, 2 ml of elution buffer containing 10% (v/v) α-methylmannoside was added to the column, and the eluent was incubated at 37 °C for 30 min. The column was then washed with 30 ml of α-methylmannoside-supplemented elution buffer at 4 °C.

**Determination of in Vivo GRP94 Phosphorylation—** Two-dimensional isoelectric focusing/SDS-PAGE of porcine GRP94 was performed using the MiniPROTEAN II system (Bio-Rad) according to the manufacturer’s instructions.

For in vivo labeling experiments, 2 × 106 J557L plasmacytoma cells were incubated for the indicated times at a concentration of 2 × 107 cells/ml in phosphate-free DMEM with 10% (v/v) fetal bovine serum and 200 μM [32P]orthophosphate. Samples were pelleted and lysed in a buffer containing 150 mM NaCl, 50 mM sodium phosphate (pH 7.4), 0.05% (w/v) SDS, 1% (w/v) Nonidet P-40, 2 mM EDTA, 50 mM NaF, 100 μM NaVO4, 1 mM phenylmethylsulfonyl fluoride, and 0.1 mg/ml soybean trypsin inhibitor for 10 min on ice. GRP94 was immunoprecipitated following pre-clearing with Pansorbin cells (Calbiochem), using 3 μl of DU120 serum and 25 μl of protein A-Sepharose beads per reaction. The beads were washed three times in 190 mM NaCl, 50 mM Tris-Cl (pH 7.4), 1.25% (v/v) Triton X-100, and 6 mM EDTA and once in phosphate-buffered saline. Beads were re-suspended in SDS-PAGE sample buffer and the eluted protein analyzed by SDS-PAGE, followed by phosphorimager quantification.

**RESULTS**

**GRP94 Co-fragmentates with β-Galactosidase through Multiple Chromatographic Steps—** Purification of GRP94 from tissue culture cells infected with rVV expressing bacterial β-galactosidase yields GRP94 containing β-galactosidase activity (data not shown). Because GRP94 certainly lacks intrinsic β-galactosidase activity, and because the two proteins copurify through multiple purification steps, we further examined these results in the context of models for GRP94-enzyme associations that are detectable in electrophoretically homogeneous GRP94 preparations.

To trace the fate of β-galactosidase during the purification of GRP94, 150 units (0.2 mg) of purified E. coli β-galactosidase was added to a pancreatic rough microsome suspension containing 240 mg of total protein, immediately prior to beginning the GRP94 purification protocol. Exogenous β-galactosidase thus represented less than 0.1% of the total protein present, by weight. GRP94 was purified from the microsomes by the method of Wearsch and Nicchitta (18); microsomes were permeabilized to obtain soluble luminal proteins, which were then
**Table I**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>mg of GRP94/mg of total protein</th>
<th>β-Galactosidase specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rough microsomes</td>
<td>0.088</td>
<td>0.614</td>
</tr>
<tr>
<td>Luminal proteins</td>
<td>0.126</td>
<td>0.724</td>
</tr>
<tr>
<td>ResourceQ</td>
<td>0.886</td>
<td>2.43</td>
</tr>
<tr>
<td>Gel filtration</td>
<td>1.0*</td>
<td>3.89</td>
</tr>
<tr>
<td>ConA eluate</td>
<td>1.0*</td>
<td>0.070</td>
</tr>
</tbody>
</table>

* Both of these fractions were electrophoretically homogeneous (see Fig. 1).

Fig. 1. SDS-PAGE analysis of purified GRP94 preparations. GRP94 was purified from pancreatic rough microsomes by the method of Wearsch and Nichitta (18). Samples (3 μg) were separated on a 12.5% SDS-PAGE gel and stained by Coomassie Blue. GRP94 was purified from microsomes spiked with β-galactosidase (lane 1). This protein was further purified by ConA chromatography (lane 2). Protein from control microsomes is depicted in lane 3. A digital image of the Coomassie Blue-stained gel is depicted.

GRP94 Is Phosphorylated by a Kinase Biochemically Distinct from GRP94—In light of the observation that GRP94 co-purified with enzymatically measurable quantities of β-galactosidase, although appearing to be electrophoretically pure, we investigated the possibility that other enzyme activities attributed to GRP94 may also reflect co-purifying or directly associated proteins. Numerous investigators have reported that GRP94 can be phosphorylated; autophosphorylation of GRP94 has been reported (15), whereas other reports indicate that it is phosphorylated by casein kinase II (CKII) (23–25), Golgi apparatus casein kinase (26), a sphingosine-dependent kinase (27), or a tightly associated, Mg2+-dependent kinase (28).

To further investigate the GRP94-associated kinase activity, GRP94 was purified from two tissue sources (murine liver and porcine pancreas), using two different methods. In the first, based on the method of Srivastava (19), tissue was homogenized in hypotonic (30 mM sodium bicarbonate) buffer and centrifuged to yield a 100,000 × g soluble fraction. This fraction was then subjected to ammonium sulfate precipitation and sequential ConA glycoprotein affinity and ResourceQ anion exchange chromatography, to yield the highly enriched GRP94 fraction. The second method was that of Wearsch and Nichitta (18) and began with the generation of rough ER microsomes by the method of Walter and Blobel (22) and detergent extraction of these microsomes. The soluble luminal proteins released from the detergent extraction were then fractionated by ResourceQ anion exchange and gel filtration chromatography.

The resulting four preparations were assayed for apparent autophosphorylation activity. Following incubation with [γ-32P]ATP, GRP94 phosphorylation was evident in all four protein preparations, although the level of phosphorylation varied by 21-fold between the preparations (Fig. 2). We compared this kinase activity to that of purified CKII, for which GRP94 is a substrate in vitro (23). 2.4 μg of GRP94 purified from a liver whole tissue extract was phosphorylated at a rate equivalent to that predicted for 3.90 milliunits, or 2.3 ng, of CKII. For GRP94 from pancreatic whole cell extract, the rate of phosphorylation was comparable with that of 0.114 milliunits, or 68 pg, of CKII. Thus, if GRP94 were a self-directed kinase, it would have a specific activity 1,040–35,300 times lower than that of CKII.

To examine which domain of GRP94 was phosphorylated by the kinase present in the purified GRP94 fractions, phosphorylation assays were performed using recombinant GRP94 fragments encoding either the carboxyl-terminal 187 amino acids of GRP94 (C[187]), or amino acids 22–337 (N[22–337]), which comprises the amino-terminal domain. No phosphorylation was observed in the N[22–337] domain, with or without full-length GRP94 present (Fig. 3). The C[187] domain was phosphorylated only in the presence of purified, full-length GRP94, and, when both substrates were available, C[187] was phosphorylated preferentially over full-length GRP94. We conclude that the carboxyl terminus is an in vitro target of the GRP94-associated kinase.

We next investigated the substrate specificity of the kinase. In assays combining full-length GRP94 and casein, GRP94 was phosphorylated and casein was not (Fig. 4A). By contrast, casein was efficiently phosphorylated by picogram quantities of CKII, clearly indicating that the GRP94 kinase was not CKII (Fig. 4A). Similar to the CKII nucleotide substrate specificity, however, phosphorylation of full-length GRP94 could be inhibited by addition of a 100-fold excess of unlabeled ATP or GTP, but was unaffected by the Hsp90 inhibitors geldanamycin and radicicol (data not shown). To examine whether GRP94 associates specifically with the kinase, GRP94 was incubated with a
molar excess of a kinase substrate, C \([187]\), or a nonsubstrate, casein. GRP94 was separated from the other proteins by ConA binding and tested in a kinase assay. If the kinase were bound to GRP94, we reasoned that it would release from GRP94 to bind C \([187]\), thus decreasing the GRP94-associated kinase activity. However, although we observed a 50% decrease in kinase activity following ConA binding and elution, no additional decrease in kinase activity was observed upon incubation with excess substrate or nonsubstrate protein (Fig. 4B). We conclude that the association between GRP94 and the kinase is not disrupted by kinase substrate, although further purification of GRP94 on ConA does decrease the kinase activity.

That C \([187]\) was phosphorylated but itself possessed no GRP94 kinase activity provided a strategy for investigating the kinase. Thus, kinase activity, assayed as C \([187]\) phosphorylation, and GRP94, assayed by immunoblot analysis, were examined during the purification process to determine whether they co-purify. Following fractionation of soluble ER luminal proteins on a ResourceQ anion exchange column, GRP94 and kinase activity were recovered in the same fractions (Fig. 5A). However, when the GRP94/kinase peak fractions were pooled and further fractionated over a heparin affinity column, they eluted in different fractions (Fig. 5B). No kinase activity was observed in the fractions containing GRP94.

We felt it was important to determine whether GRP94 phosphorylation occurs in vivo, as our observations to this point had been based on in vitro work only, and because it was unclear whether a GRP94 kinase exists in the ER. To address this question, the phosphorylation state of native pancreas-derived...
GRP94 was examined by two-dimensional isoelectric focusing/SDS-PAGE (Fig. 6A). As depicted in Fig. 6A, GRP94 could be resolved into at least two forms, as distinguished in the isoelectric focusing dimension, consistent with the presence of populations of GRP94 differing by one or more phosphate groups. Further evidence of in vivo GRP94 phosphorylation was provided by labeling tissue culture cells with [γ-32P]orthophosphate and subsequently examining the phosphorylation state of GRP94. Resolution of anti-GRP94 immunoprecipitates by SDS-PAGE and phosphorimager analysis revealed a single, prominent radioactive band corresponding to GRP94 (Fig. 6B). Treating the cells with geldanamycin, radicicol, or thapsigargin prior to or during 32P labeling did not affect the level of GRP94 phosphorylation (data not shown).

**GRP94 and Aminopeptidase Activity Are Biochemically Distinct**—A recent report proposed that GRP94 possesses aminopeptidase activity and that GRP94 is a self-directed protease (16). Methods similar to those used above to delineate GRP94 from a GRP94-directed kinase were used to examine the relationship between GRP94 and the aminopeptidase activity. Analyses were performed using the aminopeptidase substrate Ala-pNA, previously reported to be a high activity substrate for GRP94-associated aminopeptidase activity (16). Importantly, we observed that the four GRP94 preparations (murine liver and porcine pancreas, purified from ER rough microsomes and whole cell 100,000 × g supernatant) all displayed aminopeptidase activity, although the specific activity varied by 18-fold between preparations (Fig. 7). The observed large variation in aminopeptidase specific activities is evidence that some, or all, of the aminopeptidase activity could reflect low level contamination. When the rate of Ala-pNA cleavage by GRP94 preparations was compared with that of purified aminopeptidase M, we found that 2 µg of GRP94 purified from liver RM had aminopeptidase activity equivalent to 10.4 microunits, or 430 pg, of aminopeptidase M. GRP94 from pancreatic whole cell extract had aminopeptidase activity equivalent to 0.57 microunits, or 24 pg, of aminopeptidase M. GRP94 from porcine pancreas, purified from ER rough microsomes and J558L cells, labeled with [32P]orthophosphate for the times indicated. GRP94 was immunoprecipitated from the cell lysate using DU120, resolved by SDS-PAGE, and analyzed by phosphorimager, as described under “Experimental Procedures.”

Given the unusually slow kinetics and low specific activity of the GRP94-associated aminopeptidase activity, we considered the possibility that the reported intrinsic aminopeptidase activity was caused not by GRP94, but by traces of a copurifying peptidase. Fractionation of soluble luminal proteins on a ResourceQ column revealed a complex aminopeptidase elution profile, with the major peak eluting in fractions distinct from those containing GRP94 (Fig. 8). Although GRP94-containing fractions contained a tailing aminopeptidase activity, the majority of aminopeptidase activity eluted at lower salt concentrations. To investigate the possibility that the tailing activity represented an activity intrinsic to GRP94, the GRP94-containing fractions were pooled and chromatographed on a heparin affinity column. In this chromatography step, greater than 95% of the aminopeptidase activity present in the GRP94 pool was recovered in the heparin column flow-through, which contained no GRP94, whereas ~2% of the activity was found in eluted fractions containing GRP94 (data not shown).

To address the formal possibility that GRP94 is a very inefficient aminopeptidase, we examined the sensitivity of GRP94 and aminopeptidase activity to limited and complete proteolysis. GRP94 displays a hypersensitive proteolysis site in a region...
of 84 kDa. This trace amount likely represents GRP94 released from the microsomes following the freeze/thaw cycle that accompanies the use of stored membrane. Regardless of the detergent/trypsin treatment conditions, however, no change in aminopeptidase activity was observed (Fig. 9B).

To rule out the possibility that a smaller fragment of GRP94, not recognized by our antibody, was responsible for aminopeptidase activity, we carried out more extensive digests using purified GRP94. Exhaustive trypsinolysis, sufficient to completely fragment GRP94, did not lead to a decrease in aminopeptidase activity (Fig. 10). Only very extensive trypsinization (16 h at 37 °C) abrogated aminopeptidase activity. Thus, aminopeptidase activity does not depend on the structural integrity of the GRP94 polypeptide.

**DISCUSSION**

Beginning with highly purified GRP94, prepared from a variety of sources and by different purification methods, we observed a number of apparently intrinsic enzyme activities, two of which had been previously attributed to GRP94. These activities included ß-galactosidase, GRP94 COOH terminus-directed kinase activity, and aminopeptidase (15, 16). We investigated the possibility that GRP94 co-purifies with trace levels of polypeptide contaminants and that this phenomenon accounts for the observed activities. Upon further purification, GRP94 could be resolved from the noted activities, thus demonstrating that they were not intrinsic to GRP94.

In purifying any protein, it is crucial to consider what the protein will be used for, and what level of purity is required for that use. If enzymatic properties of the protein are to be examined, it must be pure enough that the data reflect properties of the protein of interest, and not those of contaminating proteins.
GRP94 was provided to elicited macrophages and B3Z T-cell hybridomas, which themselves express β-galactosidase under the IL-2 promoter (32). β-galactosidase activity was observed. Because the aforementioned rVV clone expresses β-galactosidase on a late promoter (as a marker for cloning), the GRP94 purified from these cells contained trace levels of β-galactosidase (data not shown). The data reported here thus cast into significant doubt the magnitude of the reported ovalbumin re-presentation reported in that study. The central reported observation, that necrotic cells release GRP94 and the fraction containing GRP94 is immunogenic, is likely correct. In the context of the present study, however, the source of the immunogenic activity cannot unequivocally be ascribed to GRP94 and will be discussed later.

We observed that the GRP94 kinase is distinct from GRP94 and specific for the COOH terminus. Because phosphorylation occurs in vitro, and because anion exchange and heparin chromatography revealed a single peak of COOH-terminus-directed kinase activity, we conclude that there may be a single ER luminal kinase that phosphorylates GRP94 in cells. The kinase activity bears some similarity to CKII, but is distinct from this kinase. CKII acts on GRP94 in vitro and in crude cell extracts (23, 24), and has been reported to co-purify with GRP94 (15, 24), although not following ConA chromatography (15). Like the kinase we describe, it can use both ATP and GTP as substrates (28). CKII can phosphorylate GRP94 at six putative sites, including two in the N(22–337) domain and four in the C(187) domain, with roughly equal levels of phosphorylation in the two domains (23). In contrast, we observed phosphorylation almost exclusively on C(187). Finally, we tested the ability of GRP94-associated kinase to phosphorylate casein, the sine qua non of casein kinase, and saw none, despite robust phosphorylation by purified CKII. We therefore conclude that the GRP94 kinase is a non-GRP94, non-CKII, GRP94-specific kinase that may have an as yet unidentified physiological relevance to GRP94 function.

A recent report (16) indicates that GRP94 is an aminopeptidase capable of self-proteolysis and the trimming of peptides suitable for loading onto MHC class I molecules. Although there is ample evidence for the existence of at least one ER-resident aminopeptidase (33–36), our data demonstrate that this peptidase is not GRP94. Fractionation of ER luminal proteins by ResourceQ anion exchange chromatography shows a complex elution pattern that suggests multiple aminopeptidase activities. However, there is no activity peak that co-elutes with GRP94. When GRP94-containing fractions were further fractionated by heparin chromatography, ~95% of remaining aminopeptidase activity was recovered in the flow-through, whereas the GRP94 remained resin-bound. In context of the methodologies for protein purification, such behavior is clearly indicative of a contaminating activity(s). The principles of protein purification dictate that, when purifying an enzyme, each step must increase (or leave unchanged) the ratio of enzyme activity to total protein. However, in each of these steps, we observed the precise opposite; the specific activity of the aminopeptidase decreased.

Several other aspects of the aminopeptidase properties of GRP94 bear some discussion. Site-directed mutagenesis of amino acid 655 of GRP94 has been reported to lead to decreased self-cleavage and decreased leucinyl-p-nitroanilide cleavage (16). At least two hypotheses can be offered in explanation of these results. One, GRP94 is an aminopeptidase and thus the mutation adversely affects catalysis. Alternatively, it is possible that the mutation alters the protease-hypersensitive site, rendering it less susceptible to proteolytic attack. Moreover, if this site is part of a domain that mediates interactions with

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**Fig. 10. Aminopeptidase activity in purified GRP94 is resistant to extensive proteolysis.** Purified GRP94 (from liver rough microsomes) was treated with trypsin, which was then inactivated by addition of soybean trypsin inhibitor. GRP94 was either untreated (lane 1), treated with 0.1 mg/ml trypsin (lane 2) or 0.5 mg/ml trypsin (lane 3) for 15 min on ice, or treated with 0.5 mg/ml trypsin at 37 °C for 18 h (lane 4). Lane 5 depicts trypsin (0.5 mg/ml) alone after 15 min on ice, and lane 6 depicts trypsin (0.5 mg/ml) alone after 18 h at 37 °C. A, GRP94 is digested extensively by 0.5 mg/ml trypsin in 15 min on ice. Samples were subjected to SDS-PAGE and Coomassie Blue staining. B, aminopeptidase activity persists in all treatments except 0.5 mg/ml trypsin at 37 °C for 18 h. In duplicate, samples were tested for aminopeptidase activity by incubating with Ala-pNA at 37 °C for 16 h in the dark. The average A405 and standard deviations are depicted.

(30). This is a particularly relevant consideration when dealing with GRP94 and other protein chaperones. It has been observed that “it is the function of chaperones to bind other molecules” (21), and Hsp90, the cytosolic paralog of GRP94, has been characterized as “probably one of the ‘stickiest’ proteins of the cytosol, a kind of ‘molecular glue’ ” (31). Thus, it is possible that a subpopulation of GRP94 associates with (poly)peptides in whole cell or luminal protein extracts, and that some of the enzyme activity observed is attributable to these escorted proteins. It is also possible that the enzymes are simply similar to GRP94 in the biochemical and biophysical properties used for purification. In the experiments reported herein, the latter appears to be the case for β-galactosidase.

GRP94 purification via anion exchange and gel filtration also efficiently purifies β-galactosidase. This recent observation is of particular relevance to a recent publication from this laboratory (17). In this publication, we described experiments wherein tissue culture cells were infected with a rVV expressing the immunodominant peptide epitope of ovalbumin, as a minigene construct. The GRP94 purified from the rVV-ovalbumin-infected cells was used in experiments designed to examine the re-presentation of the ovalbumin-derived peptide by antigen-presenting cells. Indeed, when this preparation of
other proteins, the mutation may lead to less association, and, therefore, decreased co-fractionation, with the aminopeptidase.

The finding that GRP94 acts as a self-directed protease would not be without precedent. There are several families of self-cleaving proteases in mammals, including the inteins, glycosylasparaginases, pyruvyl enzyme precursors, hdeghog proteins, and nucleolin (37–39). However, each of these cases is notable for specific cleavage events limited to one or two sites, denoted by specific sequence motifs. As reported by Menoret and co-workers (16), the cleavage of GRP94 at dozens of sites, if catalyzed by GRP94 itself, would be unprecedented. More plausible is the explanation that GRP94 co-fractionates with traces of an unidentified protease capable of cleaving GRP94, chromogenic substrates, and peptides suitable for MHC class I loading.

We have shown that enzyme activities can co-purify with GRP94 at biochemically significant levels. Could such quantities of contaminating proteins also be immunologically significant, i.e. capable of influencing an immune response? We suggest that the quantities of co-purifying proteins present in typical GRP94 preparations may indeed be immunologically significant. By assuming 100% peptide occupancy in GRP94 and quantitative APC uptake and re-presentation, Srivastava (7) estimates 30 × 10⁶ antigenic peptides are delivered and presented by dendritic cells in a 1-μg dose of GRP94, and suggests that even one one-hundredth of this level of delivery is sufficient to elicit a T-cell response in mice. To put these calculations in context of the purification data, we calculate that even the most enriched of our GRP94 preparations (lowest peptide activity) contain 26 × 10⁶ molecules of aminopeptidase M-like protein/μg of GRP94, assuming an aminopeptidase molecular mass similar to that of aminopeptidase M (280 kDa). Likewise, our most enriched preparations of GRP94 contain 125 × 10⁶ molecules of CKII-like protein/μg of GRP94. Continuing in this vein, purification of GRP94 from an ER microsome preparation in which β-galactosidase was initially present at a <0.1% contamination level, yielded a GRP94 preparation containing ~473 × 10⁶ molecules of β-galactosidase/μg of GRP94. Indeed, 30 × 10⁶ molecules of contaminating protein in 1 μg of GRP94 is equivalent to a mere 1:100,000 contamination, or 99.999% purity. At this low level of contamination, numerous other proteins could be present in GRP94 preparations, all of which would be considered, by biochemical criteria, to be undetectable. Efforts are currently under way to investigate the contributions of bound peptides and copurifying (poly)peptides in the immunological response to GRP94 immunization.

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REFERENCES