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Autophosphorylation of grp94 (Endoplasmin)*

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The 94-kDa glucose-regulated protein (endoplasmin, grp94) is an abundant member of the 90-kDa molecular chaperone family in the endoplasmic reticulum. We have found earlier that the 50% homologous 90-kDa heat shock protein, hsp90, has ATP-binding site(s) and autophosphorylating activity (Csermely, P., and Kahn, C. R. (1991) *J. Biol. Chem.* 266, 4943–4950). In the present paper we demonstrate that highly purified grp94 is also able to autophosphorylate itself on serine and threonine residues. grp94 can be freed from the co-purifying casein kinase II by concanavalin A affinity chromatography, and its phosphorylation is unaffected by activators and inhibitors of numerous protein kinases known to associate with the homologous hsp90. The autophosphorylation persists in immunoprecipitates and in SDS-polyacrylamide gel-purified and renatured grp94. Autophosphorylation displays a monomolecular kinetics, is activated by micromolar calcium concentrations, has an extreme heat stability, and can utilize both ATP and GTP with relatively high k_m values of 243 ± 14 μM and 116 ± 23 μM, respectively. Sequence analysis of grp94 shows the presence of two ATP-binding sites. The major product of limited proteolysis of grp94 by chymotrypsin or papain is an N-terminal 85-kDa fragment that can bind to ATP-agarose but does not show autophosphorylation. Our data suggest that grp94 has an enzymatic function analogous in many respects to the similar activity of hsp70, hsp90, and grp78 (BiP). Autophosphorylation may participate in/regulate the complex formation of these proteins, so it may be involved in their chaperone function.

Exposure of cells to glucose starvation and calcium ionophores stimulates the synthesis of a specific set of proteins localized within the mitochondria, endoplasmic reticulum, and Golgi apparatus. These glucose-regulated proteins can be divided into two groups showing extensive homology with the 70- and 90-kDa heat shock protein families. The most abundant glucose-regulated protein, grp78[1] (BiP), and grp94 (endoplasmin, ERp99, gp96, hsp100, hsp108) are the major representatives of these two grp classes (1–3).

Recent studies began to elucidate the cellular function of grp78 and grp94. Both proteins were shown to bind the immunoglobulin heavy chain in a sequential manner, and their possible involvement in chaperoning of secretory proteins was also suggested (4, 5). grp94 (hsp100) was also shown to associate with actin filaments (6, 7), and the human homologue of grp94, gp96, was identified as a tumor rejection antigen possibly involved in the peptide loading of the MHC I complex (8, 9).

The exact mechanism of the interaction of glucose-regulated proteins with their targets is unknown. grp78-related chaperone effects have been shown to be ATP-dependent. ATP is translocated to the lumen of the endoplasmic reticulum (10) and binds to grp78 (11, 12). ATP converts grp78 oligomers to the monomeric form (13) and dissociates grp78 from the immunoglobulin heavy chains (14). The maturation of immunoglobulins can be blocked either by depleting the cellular ATP levels or by mutations at the grp78 ATP-binding site (15, 16). grp78 is able to autophosphorylate itself on Thr-229 (16, 17).

In an earlier study we demonstrated that hsp90, a 50% homologous cytoplasmic counterpart of grp94, possesses ATP-binding site(s) and the ability to phosphorylate itself on serine residue(s) (18). hsp90 was shown to undergo large conformational changes after ATP addition (19), and trypanosome hsp90 seems to display an ATPase activity (20). ATP binding of grp94 was also demonstrated (10, 21). These findings raised the possibility that grp94 is also able to autophosphorylate itself. In the present paper we verified this hypothesis, showing a self-induced transfer of the γ-phosphate of ATP or GTP to serine and threonine residues of grp94, and demonstrated the presence of two ATP-binding sites on the protein. Autophosphorylation of grp94 may participate in/regulate the complex formation of this protein,[2] so autophosphorylation may be involved in the chaperone function of grp94.

MATERIALS AND METHODS

Chemicals—The chemicals used for polyacrylamide gel electrophoresis were from Bio-Rad; ready-made polyacrylamide gels and a silver stain kit were obtained from Daiichi Pure Chemicals Co. Ltd. Fine Biochemicals (Tokyo, Japan). Hydroxyapatite was an Econo-Pac HTP column from Bio-Rad. Chromatography media and protein G-Sepharose were purchased from Pharmacia Biotech Inc. Protein kinase inhibitors H-7 and H-8 were kindly provided by Dr. H. Hidaka (Nagoya University). Casein kinase II was purified as described earlier (22). Histone H1 was from Boehringer Mannheim. Omnisorb is a Calbiochem product. L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin was from Worthington. [γ-^{32}P]ATP (185 TBq/mmol) and [γ-^{32}P]GTP (185 TBq/mmol) were products of Amersham Corp. [α-^{32}P]ATP (111 TBq/

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[1] The abbreviations used are: grp78, the immunoglobulin heavy chain-binding protein (BiP); ConA, concanavalin A; DnaK, the hsp70 homologue of *Escherichia coli*; grp94, 94-kDa glucose-regulated protein (endoplasmin, ERp99, gp96, hsp100, hsp108); H-7, 1-(5-isoquinolylsulfonyl)-2-methylpiperazine; H-8, *N*-(2[methylamino]ethyl)-5-isoquinoline-sulfonamide; hsp70, 70-kDa heat shock protein and its constitutive homologue, hsc70; hsp90, 90-kDa heat shock protein; PAGE, polyacrylamide gel electrophoresis; MES, 4-morpholineethanesulfonic acid.

[2] P. Csermely, Y. Miyata, and I. Yahara, unpublished observations.

mmol) was from DuPont NEN. All of the other chemicals used were from Sigma.

Isolation of hsp90 and grp94—The 90-kDa heat shock protein was isolated from livers of 4-month-old male Sprague-Dawley rats using the method of Yonezawa *et al.* (23) as described earlier (18). The purity of this preparation was higher than 95% (usually higher than 98%) as judged by densitometry of Coomassie Blue-stained SDS slab gels (24). Protein concentrations were determined using the methods of Bradford (25).

grp94 was purified both from the mouse lymphoma cell line, L5178Y, and from livers of 4-month-old male Sprague-Dawley rats with sequential column chromatography steps including DEAE-cellulose DE52, hydroxyapatite, Sephacryl S-300 gel filtration, and Mono Q fast protein liquid chromatography, as described previously (7). To remove the traces of co-purifying casein kinase II, grp94 was further purified by concanavalin A-Sepharose affinity chromatography. The ConA-Sepharose column was equilibrated with a buffer containing 20 mM Tris·HCl, 20 mM NaCl, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, pH 7.4. Bound proteins were washed with the same buffer containing 1 M NaCl. grp94 was eluted with a linear gradient (0–1,000 mM) of α-methyl-D-mannoside. The fractions containing grp94 were collected, concentrated using a Mono Q fast protein liquid chromatography column with a 100–1,000 mM linear gradient of NaCl in a buffer of 50 mM Tris·HCl, 2 mM EDTA, 1 mM dithiothreitol, 10% glycerol, pH 7.4, and dialyzed against a buffer containing 20 mM Tris·Cl, 100 mM NaCl, 0.1 mM dithiothreitol, 10% glycerol, pH 7.4. ConA-Sepharose-purified grp94 proved to be essentially homogeneous as judged by SDS-PAGE followed by silver staining.

Detection of Casein Kinase II Activity—Casein kinase II activity of grp94 fractions was detected by phosphorylating the specific peptide substrate, RRREEETEEE, and by an intragel phosphorylation assay using dephosphorylated casein as a substrate as described previously (22).

V8 Peptide Maps of grp94—15 μg of ^{32}P-labeled grp94 was subjected to SDS-PAGE (24), and the 94-kDa band was cut from the gel. The gel piece was incubated with 300 μl of buffer (125 mM Tris·HCl, pH 6.8, 1 mM EDTA, 1 mM 2-mercaptoethanol, 0.1% SDS) for 15 min at room temperature. The remaining solution was removed, and the gel piece was homogenized in 50 μl of the above buffer plus 20% glycerol, 0.0001% bromphenol blue. Homogenized samples were loaded on a 4–15% SDS-polyacrylamide gel. On top of the samples 10 μl of 1 mg/ml V8 protease was overlaid, and the electrophoresis was performed at 15 mA constant current until the front approached the interphase between the stacking and resolving gels. The current was then stopped, and the gel was stayed 30 min at room temperature to complete the digestion. After completion of the second run the gel was stained, dried, and subjected to autoradiography.

Phosphorylation of grp94—In phosphorylation assays 1.5 μg of ConA-Sepharose-purified grp94 was incubated in 50 mM Hepes buffer, pH 7.4, at 37 °C for 20 min in the presence of 5 mM $CaCl_2$ and 400 kBq of 0.1 mM [γ-^{32}P]ATP. In some experiments $MgCl_2$ or $MnCl_2$ was used instead of $CaCl_2$, and [γ-^{32}P]ATP was substituted with [γ-^{32}P]GTP. For the characterization of the autophosphorylation, the pH of the reaction medium and final concentrations of $CaCl_2$ (or $MgCl_2$) and ATP (or GTP) were also varied as indicated in the figure legends. The reaction was stopped with boiling for 5 min in Laemmli sample buffer, and samples were subjected to SDS-PAGE (24) and autoradiography.

Immunoprecipitation of hsp90 and grp94 and Autophosphorylation of the Immunoprecipitates—Production of rabbit polyclonal anti-mouse hsp90 antibodies was described previously (6). Mouse grp94 was purified as described above, and rabbit polyclonal antibodies were raised against the purified grp94. The specific reactivity of the antibodies with rodent grp94 was confirmed by Western blotting with total cell lysates of mouse L, Hepa-1, and L5178Y cells (data not shown). Mouse Hepa-1 cell lysates were prepared in HEDG buffer (25 mM Hepes, 1.5 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, pH 7.6) as described previously (26), and 600 μl of the lysates was precleared by incubation at 4 °C for 60 min with 40 μl of Omnisorb pellet (a *Staphylococcus aureus* cell suspension expressing protein G), 40 μl of Sepharose beads, and 10 μl of nonimmune rabbit serum. After centrifugation, supernatants were mixed with 10 μl of anti-hsp90 or anti-grp94 antibodies. Mixtures were incubated for 3 h at 4 °C, and then 40 μl of protein G-Sepharose was added. After rotation at 4 °C for 60 min, immunocomplexes were washed 5 times with 800 μl each of a washing buffer (50 mM Tris·Cl, 100 mM NaF, 50 mM NaCl, 2 mM EDTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 10% glycerol, 1% Nonidet P-40, pH 8.0) and finally suspended in 50 mM Tris·Cl, pH 7.4.

10 μl of the immunocomplex beads was incubated in a buffer containing 30 mM Hepes, 400 kBq of 0.2 mM [γ-^{32}P]ATP, pH 7.5, in the presence of 5 mM $CaCl_2$ or $MgCl_2$ at 37 °C for 30 min with occasional mixings. The reaction was terminated by adding Laemmli SDS sample buffer (24) and boiling for 5 min. The proteins eluted from the immunoaffinity resins were analyzed by SDS-PAGE (24) and autoradiography.

In Situ Phosphorylation of hsp90 and grp94—4 μg of rat liver hsp90 or ConA-Sepharose-purified grp94 was applied to SDS-PAGE (24). hsp90 and grp94 bands were visualized by incubating the gel in 4 M sodium acetate (27), cut from the gel, and renatured by the method of Kameshita and Fujisawa (28). Gel pieces were gently rotated at 37 °C while 0.5 ml/s of the following buffers was sequentially added: 1) 6 M guanidine HCl, 50 mM Hepes, 2 mM 2-mercaptoethanol, pH 8.0 (1 h); 2) 0.04% Tween-40, 50 mM Hepes, 2 mM 2-mercaptoethanol, pH 7.4 (3 h); 3) 50 mM Hepes, pH 7.4, 2 MBq of 0.1 mM [γ-^{32}P]ATP, 5 mM $CaCl_2$ or $MgCl_2$ as indicated in Fig. 3*C*. After 1 h gel pieces were applied to the top of an SDS-polyacrylamide gel and fixed with agarose. [γ-^{32}P]ATP was removed by a second SDS-PAGE (24), and autophosphorylation of the samples was analyzed by autoradiography.

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out by the method of Cooper *et al.* (29) as described earlier (18).

Limited Proteolysis of grp94—Papain was activated prior to use by incubation in the presence of 25 mM Hepes, pH 7.4, 2 mM EDTA, and 40 mM 2-mercaptoethanol at 37 °C for 30 min. In one set of experiments 1.5 μg of grp94 was first digested with 7.5 ng of chymotrypsin or with 20 ng of papain in the presence of 50 mM Hepes, pH 7.4, at 25 °C for 5 or 15 min, respectively. After digestion, grp94 and its cleavage products were further incubated in the presence of 10 mM $CaCl_2$ or $MgCl_2$ and 0.1 mM [γ-^{32}P]ATP for 20 min at 25 °C. In the other type of experiments 1.5 μg of grp94 was phosphorylated as described above and then digested with 7.5 ng of chymotrypsin or 20 ng of papain at 25 °C for 5 or 15 min, respectively. In both types of experiments samples were finally subjected to SDS-PAGE (24) and the radioactivity of the fragments was analyzed by autoradiography.

RESULTS

Co-purification of grp94 with Casein Kinase II—Alignment of the mouse grp94 amino acid sequence with that of murine hsp90-α (*cf.* Refs. 30 and 31) reveals a 49% homology between the two proteins. Since hsp90 tightly associates with casein kinase II (22) and grp94 is a substrate of casein kinase II (32), we hypothesized that a similar association may occur with grp94. When apparently homogenous grp94 preparations (6, 7) were analyzed for casein kinase II activity, a significant amount of casein kinase II was found to be complexed with the protein (Fig. 1, *A* and *B*). To remove the co-purifying casein kinase II we utilized the high affinity binding of the mannose-rich grp94 (33) to ConA. After high salt wash and elution of grp94 from the ConA-Sepharose column, not even traces of casein kinase II activity could be detected in our preparation (Fig. 1, *A* and *B*). Furthermore, no casein kinase II could be detected in Western blots of 30 μg of ConA-purified grp94 with a polyclonal anti-casein kinase II antibody (kindly provided by M. Dahmus, University of California at Davis), and immunodepletion of casein kinase II did not diminish the calcium-dependent phosphorylation of grp94 (data not shown).

Phosphorylation of grp94—Since grp94 binds ATP (10) and the homologous cytoplasmic protein, hsp90, is able to autophosphorylate itself (18), we asked whether highly purified grp94 could also be phosphorylated. Our data shown in Fig. 2 confirmed our expectation. Casein kinase II-free grp94 can be phosphorylated both by ATP and GTP. Phosphorylation occurs in the presence of Ca^{2+}, Mg^{2+}, or Mn^{2+} cations; however, the extent of phosphorylation was slightly diminished using calcium-GTP (Fig. 2). When the phosphorylation reaction was performed with [α-^{32}P]ATP, labeling of grp94 was significantly reduced, suggesting that a real transfer of the ATP-(GTP)-γ-phosphate occurred (Fig. 2). Phosphorylation was time-dependent, resulting in the labeling of 2–6% of total grp94 under regular (suboptimal) assay conditions. Analysis of the phosphorylation of grp94 in the presence of either Ca^{2+} or Mg^{2+} on native gels revealed an approximately even distribution of

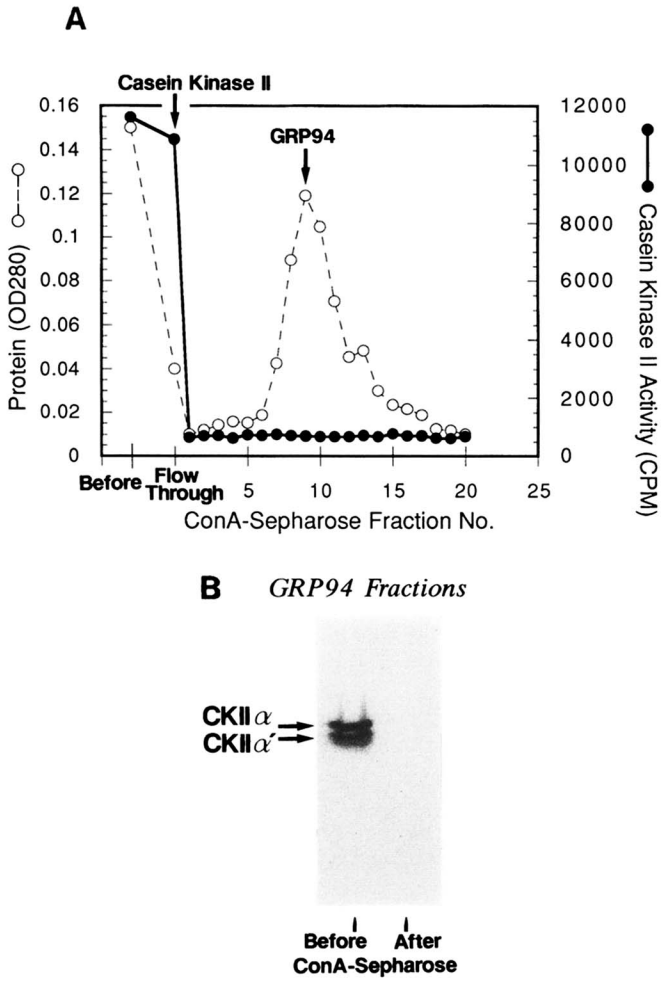

FIG. 1. **Removal of co-purifying casein kinase II from grp94 by concanavalin A affinity chromatography.** grp94 was purified as described earlier (7) with minor modifications detailed under "Materials and Methods." Afterwards the Mono Q column grp94 was further purified on concanavalin A-Sepharose as described under "Materials and Methods." Fractions were analyzed for casein kinase II activity using the peptide substrate RRREEETEEE (*panel A*, *filled circles*). Casein kinase II (*CKII*) content of 1.5 μg of pooled and concentrated grp94 was also measured by an intragel phosphorylation assay described earlier (Ref. 22; *panel B*).

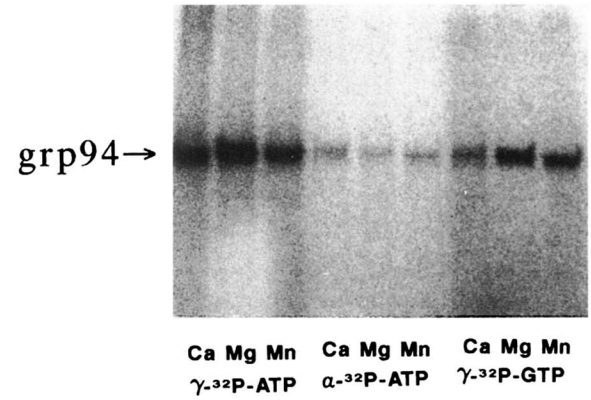

FIG. 2. **Phosphorylation of grp94 in the presence of ATP, GTP, and various divalent cations.** 1.5 μg of ConA-Sepharose-purified grp94 was incubated in 50 mM Hepes buffer, pH 7.4, at 37 °C for 20 min in the presence of 5 mM each of $CaCl_2$, $MgCl_2$, and $MnCl_2$ and 0.1 mM [γ-^{32}P]ATP/GTP as indicated. The reaction was stopped with boiling for 5 min in Laemmli sample buffer, and samples were subjected to SDS-PAGE. The autoradiogram is representative of three separate experiments.

[^{32}P]phosphate among the monomeric, dimeric, and oligomeric forms of grp94 (data not shown).

grp94 was immunoprecipitated from mouse Hepa-1 cell lysates by a rabbit polyclonal anti-grp94 antibody. Immunoprecipitated grp94 retained its ability to incorporate [^{32}P]phosphate from ATP in the presence of $CaCl_2$ even after extensive washing of the immunoprecipitates, which further suggests that the phosphorylation is an intrinsic property of grp94 and provides an easy method to assess grp94 autophosphorylation from whole cell lysates. The autophosphorylation of hsp90 immunocomplexes is also shown for comparison (Fig. 3*A*).

hsp90, a homologous cytoplasmic counterpart of grp94, has an extremely high tendency to associate with other proteins, with protein kinases (18) among others. Therefore, in spite of the apparent homogeneity of our grp94 preparation, we analyzed whether inhibitors or activators of protein kinases known to interact with hsp90 affect the phosphorylation of grp94. Phosphorylation of ConA-Sepharose-purified grp94 in the presence of Ca^{2+} or Mg^{2+} was not significantly affected by 5 μg/ml heparin, 10 μM hemin, 100 μM H-7, and 100 μM H-8, which modify the activity of casein kinase II, heme-regulated eIF-2-α kinase, protein kinase C, and cyclic nucleotide-dependent protein kinases, respectively. 50 μg/ml double-stranded DNA, an activator of the double-stranded DNA-activated protein kinase did not influence the magnesium-dependent phosphorylation of grp94; however, it induced a 90% inhibition of the phosphate transfer in the presence of calcium-ATP (data not shown). Lysine-rich histones (type III-S, Sigma) or histone H1 induced an 8–10-fold increase in the magnesium-dependent phosphorylation of grp94. On the contrary, they did not influence the calcium-dependent phosphorylation of the protein similarly to the effects of lysine-rich histones on the phosphorylation of hsp90 (Ref. 18 and data not shown).

To gain further evidence that the calcium-dependent phosphorylation is not caused by the presence of enzymatically and immunologically undetectable traces of casein kinase II, we compared the V8 peptide maps of ^{32}P-labeled ConA-purified grp94 phosphorylated in the presence of $CaCl_2$ or $MgCl_2$ without and with exogenous casein kinase II, respectively. The phosphorylation pattern of the two peptide maps was clearly different, providing further evidence that the calcium-dependent phosphorylation of grp94 was not caused by traces of contaminating casein kinase II (Fig. 3*B*).

To analyze further whether the phosphorylation of grp94 is an intrinsic property of the protein we purified ConA-Sepharose-purified grp94 using SDS-polyacrylamide gel chromatography. After SDS-PAGE and renaturation the calcium-dependent phosphorylation of grp94 still persisted. However, the magnesium-dependent phosphorylation of grp94 was significantly diminished after gel purification of the protein, similarly to that of hsp90 (Fig. 3*C*).

Phosphoamino acid analysis of phospho-grp94 revealed that the phosphorylation of the protein resulted in the transfer of [γ-^{32}P]phosphate of ATP to serine and threonine residues in the presence of $MgCl_2$ or $CaCl_2$ (Fig. 3*D*).

Characterization of grp94 Autophosphorylation—The extent of the calcium-dependent phosphorylation of grp94 does not change with the dilution of the protein (Fig. 4*A*). The monomolecular kinetics of the phosphorylation provides a further argument for the intrinsic phosphorylation of grp94 in the presence of calcium-ATP.

Autophosphorylation of grp94 displays an extreme heat stability in the presence of calcium. The amount of grp94-incorporated radioactive phosphate is essentially unchanged even after an incubation of 10 min at 95 °C (Fig. 4*B*). However, after boiling for 5 min, the autophosphorylation of grp94 is greatly

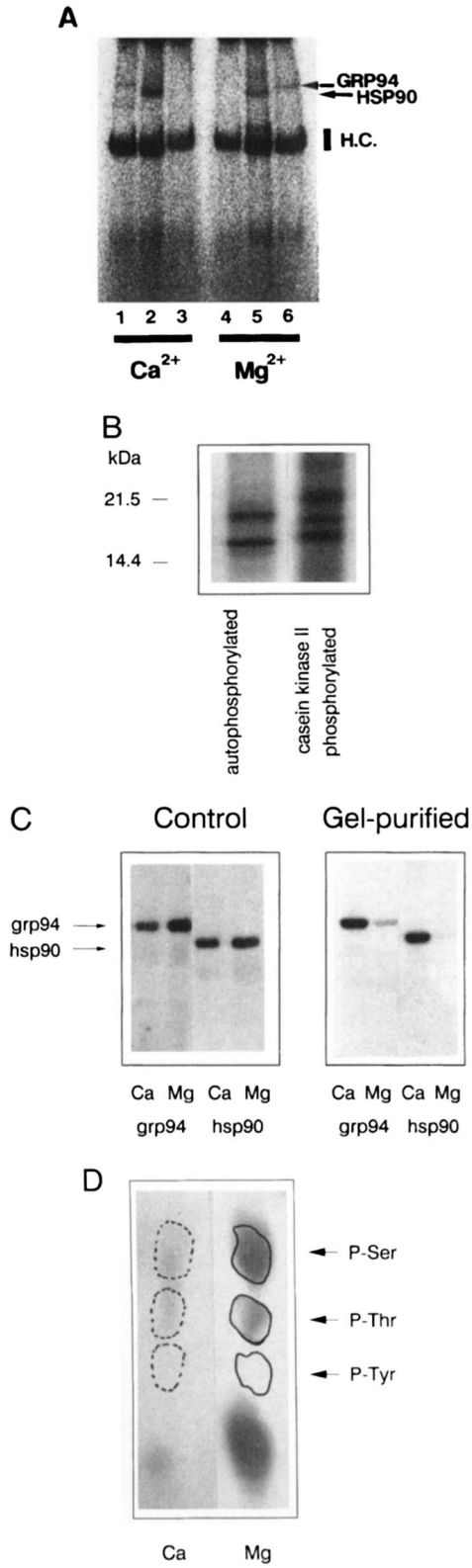

FIG. 3. **Phosphorylation of grp94 and hsp90 immunoprecipitates (*panel A*), V8 peptide map of phospho-grp94 (*panel B*), phosphorylation of gel-purified grp94 and hsp90 (*panel C*), and phosphoamino acid analysis (*panel D*).** *Panel A*, phosphorylation of grp94 immunoprecipitates. 2 mg of mouse Hepa-1 cell lysate proteins was immunoprecipitated by preimmune serum (*lanes 3* and *4*), polyclonal anti-grp94 (*lanes 1* and *6*), or anti-hsp90 (*lanes 2* and *5*) antibodies as described under "Materials and Methods." grp94 or hsp90 absorbed to protein G-Sepharose beads was incubated in the presence of 5 mM $CaCl_2$ (*lanes 1–3*) or $MgCl_2$ (*lanes 4–6*) and 0.2 mM [γ-^{32}P]ATP in 50 mM Hepes buffer, pH 7.4, at 37 °C for 30 min. Samples were subjected to SDS-PAGE (*H.C.*, immunoglobulin heavy chain). *Panel B*, V8 phosphopeptide maps of autophosphorylated and casein kinase II-phosphorylated grp94. 30 μg of ConA-Sepharose-purified grp94 was labeled with [^{32}P]phosphate in the presence of 5 mM $CaCl_2$ or $MgCl_2$ plus 1 μg of purified casein kinase II as indicated. The V8 peptide map was obtained as described under "Materials and Methods." *Panel C*, phosphorylation of gel-purified grp94 and hsp90. ConA-Sepharose-purified grp94 and hsp90 were subjected to autophosphorylation in the presence of 5 mM $CaCl_2$ or $MgCl_2$ as indicated (*Control*). Separate samples were further purified by SDS-PAGE. grp94 and hsp90 bands were cut from the gel, and after renaturation grp94 or hsp90 were phosphorylated "*in situ*" in the gel as described under "Materials and Methods." Phosphorylated proteins were subjected to a second SDS-PAGE to remove any associated [γ-^{32}P]ATP (*Gel-purified*). *Panel D*, phosphoamino acid analysis of grp94. Phosphorylation and phosphoamino acid analysis of 6 μg of ConA-Sepharose-purified grp94 was performed in the presence of 5 mM $CaCl_2$ or $MgCl_2$ as described under "Materials and Methods." Autoradiograms are representative of three (in case of *panel B*, nine) separate experiments.

diminished to 30–40% of the control value (data not shown). The magnesium-dependent phosphorylation is much more sensitive for heat denaturation, losing half of its activity at about 55 °C. In spite of this higher heat sensitivity, approximately 25% of the original magnesium-dependent activity remains stable even after a heat treatment at higher temperatures (Fig. 4*B*).

Autophosphorylation of grp94 occurs at micromolar free calcium concentrations, reaching a plateau after 10 μM Ca^{2+} (Fig. 4*C*). The magnesium-dependent phosphorylation has a sharply different pattern, activated by only millimolar concentrations of Mg^{2+} and declining after 20 mM divalent cation (Fig. 4*C*). Addition of 100 mM NaCl or KCl to the reaction medium induces a slight (approximately 20%) inhibition of both the calcium- and magnesium-dependent phosphate transfer (data not shown).

The pH dependence of the calcium- and magnesium-dependent phosphorylation of grp94 shows a broad optimum peaking around pH 7.5 and 9.0, respectively (Fig. 4*D*). On the contrary the pH dependence of calcium- and magnesium-dependent phosphorylation of hsp90 is dissimilar, rendering the calcium- and magnesium-dependent phosphorylation predominant at about pH 6.0 and 7.0, respectively (Fig. 4*E*).

The k_m values of the autophosphorylation of grp94 for ATP in the presence of $CaCl_2$ or $MgCl_2$ were 243 ± 14 μM and 111 ± 14 μM, respectively. The respective k_m values for calcium-GTP and magnesium-GTP were significantly lower, 116 ± 23 μM and 20 ± 4 μM (data not shown).

Autophosphorylation of grp94 before and after Limited Proteolysis with Chymotrypsin and Papain—Since we found two putative ATP-binding sites of grp94 (Table I), we wanted to analyze whether there is any difference in the contribution of these two sites to the autophosphorylation of grp94. Therefore we subjected our highly purified grp94 to a partial proteolytic digestion with papain and chymotrypsin. (Our repeated attempts to digest grp94 with leucine aminopeptidase were unsuccessful (data not shown).) Under the conditions described under "Materials and Methods" we gained one major proteolytic fragment migrating at about 85 kDa in SDS-polyacrylamide gel (Fig. 5). When we performed a calcium-dependent phosphorylation after the partial proteolytic digestion practically no radioactive label was associated with the 85-kDa grp94 fragment. Since the lack of labeling may have resulted from either the removal of the ATP-binding site(s) or removal of the phosphate acceptor site(s) we did the same experiment in the opposite order, first autophosphorylating grp94 and then digesting it with papain or chymotrypsin. Using the same conditions we got the same 85-kDa major proteolytic fragment. However, when performing the proteolysis after the autophosphorylation a significant labeling of

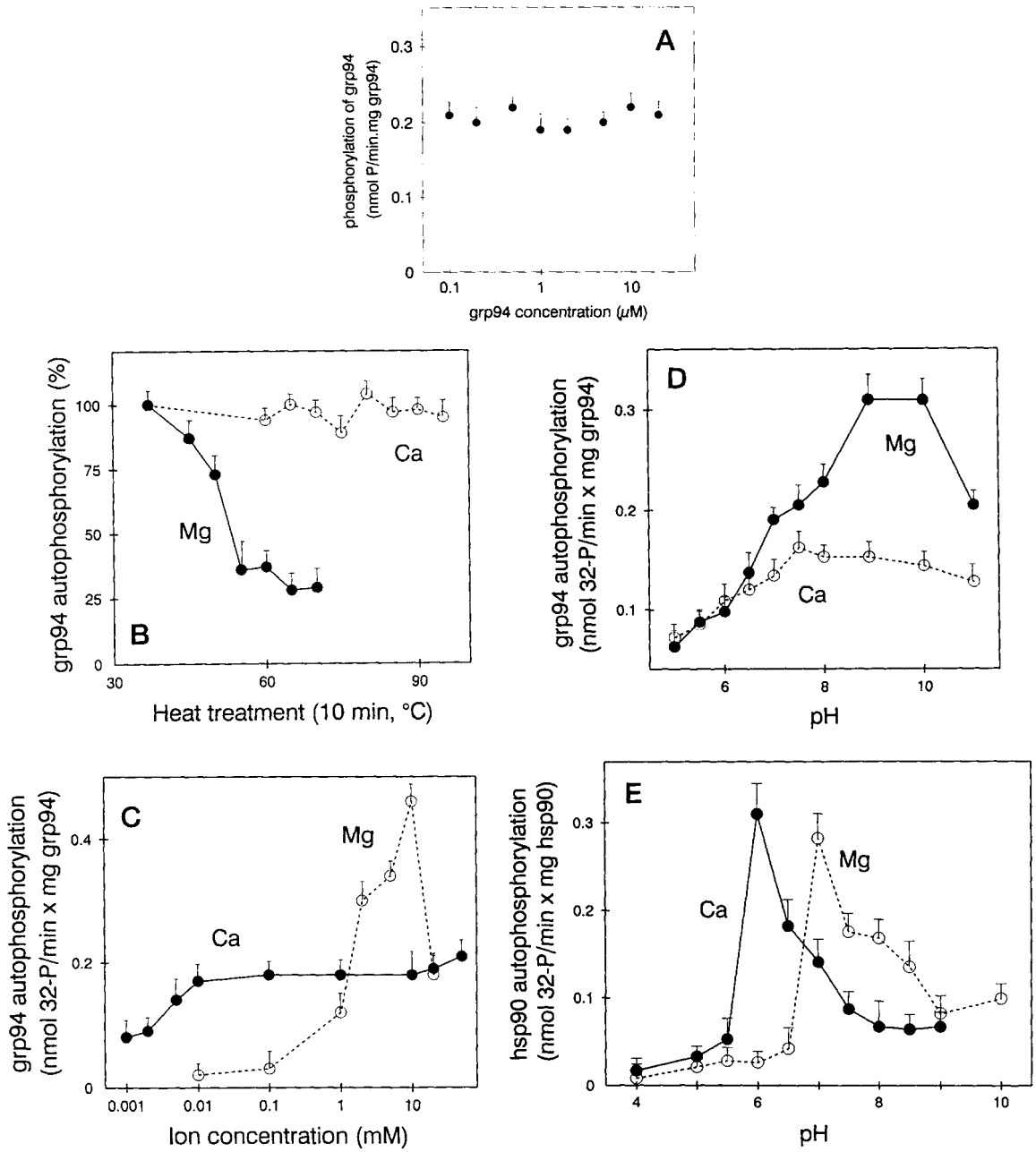

FIG. 4. **Kinetics (*panel A*), heat stability (*panel B*), ion (*panel C*), and pH (*panel D*) dependence of grp94 autophosphorylation in comparison with the pH dependence of hsp90 autophosphorylation (*panel E*).** *Panel A*, kinetics of grp94 autophosphorylation. 10 μg of ConA-purified grp94 was phosphorylated in the presence of 5 mM $CaCl_2$ after setting the final concentration of the protein as indicated under "Materials and Methods." Samples were concentrated in a SpeediVac centrifuge evaporator prior to SDS-PAGE. *Panel B*, heat stability of grp94 autophosphorylation. 1.5 μg of ConA-Sepharose-purified grp94 was incubated at the temperatures indicated for 10 min in 50 mM Hepes, pH 7.4. Samples were cooled to 37 °C and phosphorylated as described under "Materials and Methods." The control (100%) values of magnesium-dependent autophosphorylation (*filled circles*) and calcium-dependent autophosphorylation (*open circles*) were 0.4 and 0.2 nmol of ^{32}P/min × mg of grp94, respectively. *Panel C*, ion dependence of grp94 autophosphorylation. Autophosphorylation of grp94 was performed as described under "Materials and Methods" in the presence of $CaCl_2$ or $MgCl_2$ at final free concentrations indicated. Free concentrations of micromolar Ca^{2+} were set using a calcium-EGTA buffer (53). *Open* and *filled circles* represent magnesium- and calcium-dependent autophosphorylation, respectively. Data are means ± S.D. of the densitometric analysis of autoradiograms from three separate experiments. *Panels D* and *E*, pH dependence of the autophosphorylation of hsp90 and grp94, respectively. Rat liver hsp90 and ConA-Sepharose-purified grp94 were autophosphorylated as described under "Materials and Methods" in the presence of 5 mM $CaCl_2$ or $MgCl_2$. The pH of the reaction medium was set using 50 mM MES (pH range 5–6.5), Hepes (pH range 6.5–8), and Tris (pH range 8–10). Autophosphorylation of hsp90 and grp94 was not significantly different in different buffers at overlapping pH values. Data represent means ± S.D. of the densitometric analysis of autoradiograms from three separate experiments.

the 85-kDa fragment occurred (Fig. 5). Repeating the experiments with magnesium-dependent autophosphorylation gave identical results (data not shown).

When applied to an ATP-agarose column, both grp94 and its 85-kDa proteolytic fragment were retained, suggesting the presence of a functional ATP-binding site in the 85-kDa fragment (in control experiments with agarose microcolumns both proteins were in the flow-through fraction (data not shown). Microsequencing the 85-kDa fragment gave an N terminus of DDEVD, which completely matches the processed N-terminal sequence of mouse grp94 (30). (There was an agreement between the sequence of the 85-kDa fragment and that of grp94 at the consecutive 8 amino acid residues as well; data not shown.) These results reflect that both papain

TABLE I
ATP binding sequences of grp94

Protein	Residues	Sequence[a]
Type A sequence[b]		
Consensus sequence (38, 39)[c]		**A/G-***X***-***X***-***X***-***X***-G-K-T/S-***X***-***X***-***X***-***X***-***X***-***X***-I/V**
gp96 (37)	10–27	
hsp108 (36)	9–26	
ERp99 (30)	31–48	V-E-E-D-L-**G-K-S**-R-E-G-S-R-T-D-D-E-**V**
gp96 (9, 37)	217–228	
hsp108 (36)	237–249	
ERp99 (30)	238–250	**G**-N-T-L---**G-R**-G-**T**-T-I-T-L-**V**
Type B sequence[b]		
Consensus sequence (38, 39)		**H/R/K----***X*$_{(5-8)}$**----O-***X***-O-O-D/E**[d]
gp96 (37)	49–60	
hsp108 (36)	68–80	
ERp99 (30)	70–81	**R**-E-K-S-E-K-F---**A**-F-**Q-A-E**
gp96 (37)	193–203	
hsp108 (36)	213–223	
ERp99 (30)	214–224	**K**-H-N-N-D-T-----**Q**-H-**I-W-E**
gp96 (9, 37)	231–241	
hsp108 (36)	251–261	
ERp99 (30)	252–262	**K**-E-E-A-S-D-----**Y**-L-E-**L-D**
gp96 (9, 37)	303–313	
hsp108 (36)	323–333	
ERp99 (30)	324–334	**K**-K-V-E-K-T-----**V**-W-D-**W-E**
gp96 (37)	465–477	
hsp108 (36)	485–497	v[e]
ERp99 (30)	486–498	**K**-E-F-G-T-N-I-K-**L**-G-**V-I-E**
gp96 (37)	687–698	l
hsp108 (36)	707–718	s
ERp99 (30)	708–719	**K**-T-V-M-D-L-A---**V**-V-**L-F-E**

[a] Gaps are marked with hyphens, and consensus sequences are in boldface type.
[b] Type A and Type B sequences denote the putative triphosphate- and adenine-binding sequences, respectively (38, 39).
[c] The numbers in parentheses denote the appropriate references.
[d] O, hydrophobic residue.
[e] Lowercase letters denote conservative replacements of the particular amino acids as compared with the sequence of ERp99.

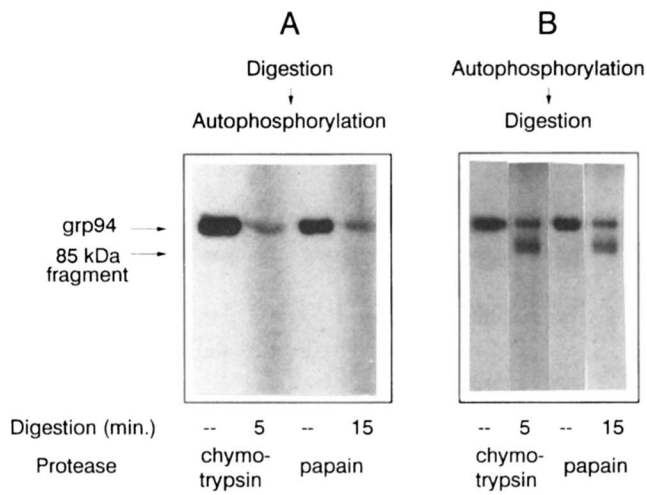

FIG. 5. **Autophosphorylation of grp94 after (*panel A*) and before (*panel B*) limited proteolysis with chymotrypsin and papain.** *Panel A*, autophosphorylation of grp94 after limited proteolysis; *panel B*, autophosphorylation of grp94 before limited proteolysis. Limited proteolysis and calcium-dependent autophosphorylation of 1.5 μg of ConA-Sepharose-purified grp94 were performed as described under "Materials and Methods." Samples were subjected to SDS-PAGE, and the radioactivity of the proteolytic fragments was analyzed by autoradiography. Autoradiograms are representative of two separate experiments.

and chymotrypsin truncate grp94 at its C terminus.

DISCUSSION

In an earlier study we demonstrated that hsp90, an approximately 50% homologous cytoplasmic counterpart of grp94, possesses ATP-binding site(s) and is able to phosphorylate itself on serine residue(s) (18). grp94 was also shown to bind ATP (10, 32). These findings raised the possibility that grp94 is also able to autophosphorylate itself.

Our experiments revealed that highly purified preparations of grp94 can incorporate radiolabeled phosphate from the γ-position of both ATP and GTP. Control experiments with α-^{32}P-labeled ATP as well as the detection of the radiolabel on serine and threonine residues after phosphoamino acid analysis strongly suggest that the γ-phosphate was transferred to grp94.

We detected a significant amount of casein kinase II copurifying with apparently homogenous grp94 preparations. This is not surprising, since the homologous hsp90 tightly associates with a number of protein kinases including casein kinase II (18, 22), and grp94 is a good substrate of casein kinase II (33). The complexing of grp94 with casein kinase II may occur *in vivo*, since grp94 was reported to be associated with the nucleus (34), and casein kinase II, a predominantly nuclear protein kinase, was also reported to be present in the endoplasmic reticulum (32). Further studies are necessary to elucidate whether this is indeed the case or whether grp94 sticks to casein kinase II during the isolation procedure.

Because of the tight association of grp94 with casein kinase II extreme care should be exercised to preclude the possibility that the phosphorylation of purified grp94 was induced by traces of contaminating casein kinase II. In our studies we obtained several lines of evidence against this possibility. 1) Casein kinase II can be efficiently removed from grp94 by ConA affinity chromatography and high salt wash of the latter protein. ConA-purified grp94 contains no casein kinase II detected by enzymatic analysis using two appropriate substrates, the peptide RRREEETEEE, and dephosphorylated casein. 2) ConA-purified grp94 does not contain any immunodetectable casein kinase II, and immunodepletion of putative traces of casein kinase II does not diminish the calcium-dependent phosphorylation of grp94. 3) The phosphorylation pattern of auto-

phosphorylated grp94 clearly differs from that of the casein kinase II-phosphorylated protein. 4) Calcium-dependent phosphorylation of grp94 cannot be inhibited by heparin, a sensitive inhibitor of casein kinase II, and displays an extreme heat stability, which is not characteristic of casein kinase II even in its complex with hsp90, a highly homologous heat shock protein (22).

Although casein kinase II activity can be efficiently and fully removed from grp94 preparations by concanavalin A affinity chromatography the possibility still persists that traces of other contaminating kinases are still present in the grp94 preparation and induce the calcium-dependent phosphorylation of the 94-kDa band. Several lines of evidence show, however, that this is not the case. 1) The phosphorylation of grp94 persists after immunoprecipitation of grp94 by anti-grp94 antibodies and after further purification of the protein on SDS-PAGE. 2) The calcium-dependent phosphorylation displays a monomolecular kinetics. 3) The approximate k_m of the reaction is 0.24 mM for ATP. This is much higher than the reported values for most other protein kinases. Furthermore, phosphorylation is not affected by a number of activators and inhibitors of the protein kinases that might associate with grp94. 4) The phosphorylation displays a unique cation dependence, being active in the presence of Ca^{2+} ions alone. 5) The activity is surprisingly heat-stable. grp94 retains its autophosphorylation activity almost fully even after incubation for 10 min at 95 °C.

Thus, these data strongly suggest that grp94 itself possesses an intrinsic, calcium-dependent autophosphorylating activity. Many of the arguments listed above are also valid for the magnesium-dependent phosphorylation of grp94. However, the magnesium-dependent phosphorylation displays a much smaller resistance against heat treatment than its calcium-dependent counterpart, and the extent of magnesium-dependent phosphorylation is significantly diminished after gel purification of grp94. On one hand these differences may reflect the involvement of remote amino acid side chains in the active center of grp94 phosphorylation in the presence of magnesium, which are parts of protein segments being more sensitive for heat denaturation and do not completely refold after guanidinium chloride treatment. On the other hand we cannot exclude the possibility that the magnesium-dependent phosphorylation of grp94 occurs as a result of a trace amount of contaminating protein kinase, which is not similar to those kinases known to be associated with hsp90.

Dechert *et al.* (35) have isolated an 80-kDa protein kinase from the microvessels of porcine brain. The protein had an N-terminal amino acid sequence similar to that of hsp108, a chicken homologue of grp94 (35, 36). Our findings further suggest that the 80-kDa protein of Dechert *et al.* (35) is indeed a grp94 homologue displaying a similar enzymatic activity. During the preparation of our manuscript Li and Srivastava (9) reported that the structure of grp94 contains an ATP-binding site and that the protein displays an ATPase activity similar to that of hsp90. In our studies we also detected a significant ATPase activity of grp94, albeit significantly smaller than that reported by Li and Srivastava (9). The ATPase activity was greatly diminished when we further purified grp94 with ConA-Sepharose affinity chromatography (data not shown). These findings substantiate the conclusion that the acceleration of phosphate transfer in highly purified grp94 preparations is an intrinsic property of grp94.

Comparing the primary structure of mouse (30), chicken (36), and human (37) grp94 homologues with ATP binding consensus sequences (38, 39), we found a second ATP-binding site of grp94 located toward the N-terminus from the ATP-binding consensus sequence identified by Li and Srivastava (Table I).

TABLE II
Comparison of the autophosphorylation of grp94, hsp90, grp78, and hsp70

Property	grp94	hsp90	grp78 (BiP)	hsp70 (DnaK)
Cation dependence				
Calcium	μM	mM (18)[a]	μM (16)	mM (52)
Magnesium	mM (?)	None (18)	None (16)	None (52)
k_m for ATP (mM)	0.24	0.16 (18)	0.001 (16)	ND[b]
pH optimum	7.5	6.5	5.5 (17)	6.0 (51)
Heat stability	95 °C	75 °C (18)	ND	100 °C (52)
Phosphorylated amino acid	Ser,Thr	Ser (18)	Thr (16)	Thr (51)
Stoichiometry (%)	6	2 (18)	4 (16)	15 (52)

[a] The numbers in parentheses denote the appropriate references.
[b] ND, not determined.

Interestingly the crucial GKT motif in the ATP-binding site of hsp90 (18) was conservatively mutated to GKR in grp94, which makes it very unlikely that these otherwise highly homologous proteins use the same ATP-binding sites.

Partial proteolytic digestion of grp94 with papain or chymotrypsin produced a major proteolytic fragment of 85 kDa. The identity of the N terminus of this fragment with that of grp94 suggests that under these conditions papain and chymotrypsin remove the C-terminal end of grp94, leaving its N terminus intact. Our difficulties in obtaining the N-terminal sequence of the 85-kDa fragment and our unsuccessful attempts to digest it with leucineaminopeptidase suggest that the N terminus of grp94 is blocked. These conclusions are in agreement with the earlier data of Kulomaa *et al.* (36) and Edwards *et al.* (40). When the last 80–90 amino acid residues were removed from grp94, the autophosphorylation sites remained intact, since when autophosphorylating grp94 prior to the partial proteolytic digestion the radiolabel was recovered in the major, 85-kDa proteolytic fragment. On the other hand, the C-terminal end of the protein may be necessary for the autophosphorylation to occur. This part of grp94 contains an adenine-binding consensus sequence (amino acids 708–719 of ERp99, Table I), which may participate in the autophosphorylation.

Autophosphorylation of grp94 requires the presence of either calcium or magnesium ions. grp94 is reported to be a calcium-binding protein (32) possessing 4 high affinity and 11 low affinity calcium-binding sites with apparent dissociation constants of 2 and 600 μM, respectively (34). Examining the primary structure of grp94 we found three conserved putative high affinity calcium-binding sites displaying a homology with the consensus sequence of the calcium binding EF-hand motif (41). Among these three sequences (amino acids 224–235, 308–319, and 440–451 in the mouse grp94 sequence (30) the second and its surroundings had an unambiguous α-helical structure, the third EF-hand region was partially α-helical, and the first EF-hand region contained hardly any α helical segments as predicted by the methods of Chou and Fasman (42) and Garnier *et al.* (43). grp94 also has a number of amino acid sequences close to its C terminus, which display a partial homology with EF-hand structures functioning presumably as low affinity calcium-binding sites. The presence of high affinity calcium-binding sites on grp94 provides a structural and functional explanation of the activation of its autophosphorylation by micromolar concentrations of calcium.

Comparing the characteristics of grp94 autophosphorylation with hsp90, grp78 (BiP), and hsp70 (DnaK) autophosphorylation, we found that both glucose-regulated proteins, grp94 and grp78, residing in the lumen of the endoplasmic reticulum show a high, micromolar affinity for calcium, whereas their cytoplasmic counterparts, hsp90 and hsp70, are activated only in the presence of millimolar concentrations of the cation

(Table II). On the contrary, cytoplasmic and luminal free Ca^{2+} concentrations are generally assumed to be approximately 0.1 and 100 μM, respectively (44). Intracellular calcium concentration is reported to be increased after various environmental stresses (45), and depletion of the calcium stores of the endoplasmic reticulum may contribute to the activation/synthesis of grp78 (46, 47). Further studies are required to elucidate whether stress-induced local perturbations in the cytoplasmic and luminal calcium concentration may induce the activation of hsp90 and grp94 autophosphorylation, respectively.

Although to the best of our knowledge the luminal free Mg^{2+} concentration has never been exactly measured, it is assumed to be in the millimolar range (48). The sharp increase in the magnesium-dependent autophosphorylation of grp94 makes the luminal Mg^{2+} concentration a good candidate for the regulation of grp94, similar to grp78 (47).

Heat shock and other stresses cause a drop in the intracellular pH and ATP concentration (49). Whereas stress-induced intracellular acidification may activate the autophosphorylation of hsp90, it is hardly playing any role in the regulation of grp94. On the contrary, the relatively high k_m for ATP makes both hsp90 and grp94 sensitive to respond to greater changes in intracellular ATP concentration after various stresses, *e.g.* ischemia.

The stoichiometry of the autophosphorylation is rather low, reaching 2–6% under regular (suboptimal) assay conditions. Incubating the protein at a higher temperature (50–60 °C) for longer times (30–60 min) increased the extent of autophosphorylation. However, the calcium-dependent autoproteolysis of grp94[3] competed with the reaction and prevented the full analysis of the stoichiometry of the reaction (data not shown). A low level of autophosphorylation (2–15%) is a characteristic feature of all heat shock proteins (Table II). Autophosphorylation may accompany the association/dissociation of heat shock proteins with their targets, which may be rate-limiting.[2]

The autophosphorylation of grp94 may occur only transiently *in vivo*. grp94 is reported to be a phosphoprotein only in some cell types (32, 34). This may indicate that its autophosphorylation sites are not constantly occupied *in vivo*. The presence of phosphoprotein phosphatases and the transfer of the γ-phosphate from serine and threonine residues of grp94 to water via an ATPase reaction may explain why Clairmont *et al.* (10) did not get a significant thiophosphorylation of grp94 in intact canine pancreas microsomes. Recent studies indicate that the autophosphorylation of grp78 may also occur only transiently *in vivo* since its autophosphorylation site, Thr-229, is usually found nonphosphorylated in isolated grp78 (17).

Since the magnesium-dependent autophosphorylation of grp94 is greatly enhanced by lysine-rich histones, *e.g.* by histone H1, and grp94 was reported to be enriched in the cell nucleus after heat shock (34), the autophosphorylation of grp94 may be involved in the protection of nuclear structures after environmental damage. The autophosphorylation of grp94 may also play a role in the dissociation of grp94 from other proteins in the analogy of similar effects on hsp60 (50), grp78 (12–14), and hsp90 (51).[2] This may reflect an involvement of grp94 autophosphorylation in the chaperoning of secretory proteins and in the peptide loading of the MHC I complex (8, 9).

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Addendum—While our paper was under review, Dechert *et al.* (54) published the full sequence of the grp94-related protein kinase of porcine brain showing a 92–98% homology with grp94. Their results are consistent with our findings on the autophosphorylation of grp94 and raise the possibility that the protein is able to phosphorylate substrates other than itself.

[3] P. Csermely, T. Schnaider, and C. Sőti, unpublished observations.

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