Interaction of Endoplasmic Reticulum Chaperone GRP94 with Peptide Substrates Is Adenine Nucleotide-independent*

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The abbreviation used are: hsp, heat shock protein; GRP, glucose-regulated protein; VSV, vesicular stomatitis virus; CKII, casein kinase II; ER, endoplasmic reticulum; PAGE, polyacrylamide gel electrophoresis; BiP, binding protein; 32P, 32P-labeled inorganic phosphate; BSA, bovine serum albumin; ConA, concanavalin A.

GRP94, the endoplasmic reticulum paralog of hsp90, has recently been identified as a peptide and adenine nucleotide-binding protein. To determine if adenine nucleotides directly contribute to the regulation of GRP94 peptide binding activity, an in vitro peptide binding assay was developed. Using purified GRP94, we observed specific, saturable, temperature-sensitive binding of the peptide VSV8, a known in vivo ligand. ATP was without effect on VSV8 binding to GRP94, whether present during or subsequent to peptide binding. To evaluate the interaction of GRP94 with adenine nucleotides, the ATP binding and hydrolysis activities were directly assayed. Only negligible binding of ATP to GRP94 was observed. In addition, analysis of the GRP94 adenine nucleotide content indicated that GRP94 did not copurify with bound adenine nucleotides. GRP94 preparations exhibited low ATPase and apparent autophosphorylation activities. Further purification, combined with inhibitor studies, indicated that both activities were the result of trace contamination (<0.1%) with casein kinase II. On the basis of these data, we propose that the peptide binding activity of GRP94 is adenine nucleotide-independent and that ATP binding and hydrolysis are not inherent properties of GRP94.

Molecular chaperones are abundant, ubiquitous proteins that participate in a variety of cellular processes, such as protein folding and assembly, protein translocation across membranes, and protection from cell stress (1–3). The highly conserved hsp60,1 hsp70, and hsp90 families of stress proteins are among the most prominent chaperones and are noteworthy for their multi-compartmental localization within the cell and broad phylogenetic distribution (1, 3). Substantial insights into the molecular mechanism of chaperone function have been obtained in studies of the hsp60 and hsp70 proteins (3–7). For these proteins, chaperone activity is expressed through cycles of (poly)peptide binding and release, the kinetics of which are regulated through the binding and hydrolysis of ATP (1, 7, 8).

The molecular basis of hsp90 function is poorly understood. GRP94, the endoplasmic reticulum (ER) hsp90 paralog, is induced by the accumulation of unfolded proteins in the ER, can be found in association with nascent polypeptides in the ER lumen, and is thus likely to display chaperone activity (9–11). The identification of GRP94 as a tumor rejection antigen of murine chemical sarcomas has led to the discovery that GRP94 is a peptide-binding protein (12–15). Furthermore, GRP94 has been demonstrated to bind and hydrolyze ATP (12, 16) and undergo autophosphorylation (17). On the basis of these observations, it would appear that GRP94, and by inference, the hsp90 family of molecular chaperones, function in a manner analogous to the hsp60 and hsp70 families of molecular chaperones.

Using a peptide substrate previously identified as an in vivo ligand (15), we report that GRP94 displays saturable, specific, temperature-sensitive peptide binding activity that is insensitive to the presence of adenine nucleotides. Because we presumed that the GRP94 ATPase activity is related to its chaperone function, the ability of GRP94 to bind and hydrolyze ATP were analyzed in parallel with BiP (GRP78), an ER-resident ATPase and ATP-binding protein (18, 19). Equilibrium ATP binding studies indicate that GRP94 is not an ATP-binding protein. Low levels of ATPase and apparent autophosphorylation activity were reproducibly observed with the purified protein. Pharmacological studies and further purification indicated that both activities reflected fractional (<0.1%) contamination with casein kinase II. We conclude that the peptide binding activity and presumably the chaperone function of GRP94 are ATP-independent.

**EXPERIMENTAL PROCEDURES**

Reagents—All chemicals were of analytical grade. Bovine IgG, Dowex 1 X 8–50 (Cl−), activated charcoal, spermidine, and polylysine were obtained from Sigma. ATP and GTP were purchased from Boehringer Mannheim. [α-32P]ATP (3000 Ci/mmol), [γ-32P]ATP (6000 Ci/ mmol), and Na2[32P]I were obtained from Amersham Corp.

Purification of GRP94 and BiP—GRP94 was purified from rough microsomes prepared by the method of Walter and Blobel (20) as described previously (21). The BiP-enriched fractions from the MonoQ stage of the GRP94 purification (21) were pooled and purified by hydroxyapatite chromatography. The BiP pool, dialyzed in 10 mM potassium phosphate, pH 6.8, 20 mM NaCl, 0.5 mM Mg(OAc)2 (buffer A) was loaded onto a 5-ml hydroxyapatite column and BiP eluted with a 40-m
dradient of 10 to 450 mM potassium phosphate, pH 6.8, in buffer A. BiP-enriched fractions were identified by SDS-PAGE, pooled, and concentrated by centrifugal ultrafiltration (Centricon-30; Amicon, Beverly, MA).

**Peptide Binding Assay**—Peptide VSV8 (RGYVYQGL), has been identified as an in vivo ligand of GRP94 (15). A peptide corresponding to the VSV8 sequence with an additional N-terminal cysteine, termed C-VSV8, was synthesized by standard Fmoc (Pierce) and unincorporated [125I]iodine removed by anion exchange chromatography on Dowex 1 X 8–60 (Cl−) resin. The specific activity of the [125I]C-VSV8 was typically 0.1–0.25 Ci/mg.

Peptide binding assays were performed as follows. GRP94 (14 μg) was incubated with 37.5 μM [125I]C-VSV8 in 100 μl of buffer B (110 mM KOAc, 20 mM NaCl, 25 mM KOH-Hepes, pH 7.2, 2 mM MgCl2, and 0.1 mM CaCl2) for 1 h at 37 °C or 10 min at 50 °C. Samples were applied to
Peptide Binding by GRP94

Peptide Binding to GRP94 in Vitro—Peptide binding to GRP94 was assayed with VSV8, the immunodominant peptide of VSV. VSV8 has been identified as a native GRP94 ligand in VSV-infected cells (15) and thus represents an appropriate peptide for in vitro binding studies. The peptide used in this study was synthesized with an N-terminal cysteine and is denoted C-VSV8. To determine if GRP94 was capable of binding C-VSV8 in vitro, purified GRP94 was incubated with \( [^{125}\text{I}]\text{-C-VSV8} \) either 1 h at 37 \(^\circ\)C and bound versus free peptide was separated by gel filtration chromatography (Fig. 1A). A peak of \( [^{125}\text{I}]\text{-C-VSV8} \) labeled peptide (fractions 7–8) was observed that co-eluted with GRP94 and was resolved from free peptide (Fig. 1A). Peptide binding was markedly stimulated at higher (50 \(^\circ\)C) temperatures, suggesting that there exists a kinetic barrier to binding, which is as yet unidentified. Binding of C-VSV8 to GRP94 was saturable with an apparent \( K_d \) of 15 \( \mu \text{M} \) (Fig. 1B). In addition to exhibiting sat-
activity of GRP94 suggests that ATP binding may not be an intrinsic biochemical property of GRP94. Because the methods used previously to assess the nucleotide binding properties of GRP94 were indirect (12, 16), the ATP binding properties of GRP94 were directly assayed. GRP94 was incubated with increasing concentrations of [α-32P]ATP for 1 h at 30 °C. Bound versus free ATP were separated by vacuum filtration. The quantity of ATP bound in the presence of a 50-fold molar excess of unlabeled ATP was subtracted as background from all points. A, Scatchard plot of the binding data. For both plots, ○ indicates BiP; and ● indicates GRP94.

Although little binding of ATP to GRP94 was observed, a scenario in which nucleotide binding was limited by exchange with endogenous nucleotide was considered. To assay for bound nucleotides, the A260/A280 ratio for purified GRP94 and BiP was determined. The ratio for GRP94 was 1.6 compared with 1.2 for BiP, suggesting that nucleotides are not found in association with native GRP94 (data not shown). In addition, the ribonucleotide content of perchloric acid extracts of GRP94 and BiP were analyzed by MonoQ chromatography and UV spectrometry. In agreement with the A260/A280 data, extracts from BiP, but not GRP94, contained bound adenine nucleotides (data not shown).

Kinetics of GRP94-associated ATP Hydrolysis—In an attempt to reconcile reports of GRP94 ATPase activity (12) with the extremely low molar stoichiometry of ATP binding (Fig. 2), the kinetics of ATP hydrolysis by GRP94 were evaluated in parallel with BiP. In these experiments, GRP94 and BiP were incubated with increasing concentrations of [γ-32P]ATP for 1 h at 37 °C, and the amount of ATP hydrolyzed, assayed as 32Pi release, was determined (Fig. 3A). Low levels of ATP hydrolysis in GRP94 preparations were detectable. Analysis of the data by Lineweaver-Burk plots indicated that the K_m for ATP was 3.4 μM for GRP94 and 28.6 μM for BiP and the V_max was 0.36 pmol/min for GRP94 and 4.76 pmol/min for BiP (Fig. 3B). These data are in general agreement with previous reports by Li and Srivastava (12) and Kassenbrock and Kelly (19) with regard to GRP94 and BiP, respectively. Interestingly, these data indicate that the turnover rate for GRP94 is 0.029 mol/min/mole, corresponding to one molecule of ATP hydrolyzed per molecule of GRP94 per 34 min. Such low values are without precedent, even with respect to the documented, sluggish ATPase activities characteristic of molecular chaperones (8).

Evidence for the Co-purification of Casein Kinase II with GRP94—To determine the origin of the ATP binding and hydrolysis activities present in our GRP94 preparations, we examined the possibility that the assayed activities were due to a low enrichment, high activity contaminant. GRP94, cytosolic hsp90, and several ER luminal proteins have previously been observed to co-purify with and be phosphorylated by casein kinase II (CKII), a messenger-independent, serine/threonine protein kinase (24–28). If CKII co-purified with GRP94, it would be predicted on the basis of the abundance of CKII phosphorylation sites on GRP94 that GRP94 would serve as a CKII substrate. Incubation of GRP94 with 10 μM [γ-32P]ATP for 1 h at 37 °C and analysis of the SDS-PAGE resolved protein by phosphorimaging indicated that GRP94 was indeed subject to phosphorylation. As depicted in Fig. 4A (lane 1), GRP94 contains covalently associated 32Pi. Quantitation against internal 32Pi standards indicated that under the described conditions 1.5–4% of the GRP94 was phosphorylated. Inhibition by heparin, stimulation by polyamines and basic polypeptides, and the ability to utilize GTP and ATP are diagnostic of CK II activity (28). When these compounds were included in the assay, the incorporation of 32Pi in GRP94 was affected in a manner consistent with the presence of CKII. Heparin at 5 μg/ml completely blocked phosphorylation (Fig. 4A, lane 4), GTP at a 50-fold molar excess reduced phosphorylation by 92%

2 P. A. Wearsch and C. V. Nicchitta, unpublished observations.
The release of 32Pi. A phosphorylation 2-fold (Fig. 4) in the presence of 500 μM GTP, 50 μg/ml polylysine, or 1 mM spermidine. ATP hydrolysis was monitored by the release of 32Pi. C. GRP94 was bound to ConA-Sepharose and extensively washed in a low salt or high salt buffer. Phosphorylation of equal amounts of control (non-ConA purified) and the ConA purified GRP94 were analyzed as described above. The data presented for each panel are representative of two separate experiments.

FIG. 4. Co-purification of CKII with GRP94. A. GRP94 was incubated with 10 μM [γ-32P]ATP for 1 h at 37 °C and analyzed by SDS-PAGE. Compounds diagnostic of CKII activity were included in the incubation at the indicated concentrations. B. GRP94 was incubated with 10 μM [γ-32P]ATP in the absence or the presence of 500 μM GTP, 50 μg/ml polylysine, or 1 mM spermidine. ATP hydrolysis was monitored by the release of 32Pi. C. GRP94 was bound to ConA-Sepharose and extensively washed in a low salt or high salt buffer. Phosphorylation of equal amounts of control (non-ConA purified) and the ConA purified GRP94 were analyzed as described above. The data presented for each panel are representative of two separate experiments.

The finding that the peptide binding activity of GRP94 was independent of adenine nucleotides led us to re-evaluate previous reports that GRP94 is an ATP-binding protein and ATPase (12, 16). The ATP binding and hydrolyzing properties of GRP94 were analyzed in parallel with BiP, a well-characterized ATPase (18, 19, 23). Although apparent binding of ATP to GRP94 was observed, the low molar stoichiometry of binding (0.026 mol of ATP bound/mol GRP94), raised the specter of a low enrichment, ATP-binding contaminant. The observation that GRP94 was phosphorylated upon incubation with [γ-32P]ATP, combined with previous reports demonstrating a propensity of CKII to copurify with hsp90 and GRP94, further implicated a contaminant protein as the underlying basis for these activities. Based on the sensitivity of the CKII ATPase and phosphorylation activities to compounds that are diagnostic of CKII activity, it was concluded that trace amounts of this enzyme co-purify with GRP94. It should be noted that the specific activity of ATP hydrolysis by CKII is quite high, with values up to 5000 pmol ATP hydrolyzed/min/μg of protein reported (29). At this specific activity, a 0.06% level of CKII contamination (GRP94 purity of 99.94%) would be sufficient to achieve the observed levels of ATPase activity and phosphorylation.

It has been established that the lifetimes of hsp60- and hsp70-substrate interactions are governed by the kinetic parameters of ATP binding and hydrolysis (7, 22, 30, 31). In the case of DnaK, the rates of ATP hydrolysis and ADP-ATP exchange are further regulated through interaction with DnaJ and GrpE, respectively (7, 32). Such regulation is reminiscent of GTP-binding proteins, whose interactions with effectors are regulated by the kinetics of GTP binding and hydrolysis (33, 34). In view of the data reported herein, indicating adenine nucleotide-independent peptide binding by GRP94, it is of value to ask whether the peptide binding activity of GRP94 is under regulatory control. It has been noted that the kinetics of ATP binding and hydrolysis of the hsp60 and hsp70 chaperones are quite well matched to the rates of cellular protein synthesis (8). By similar logic, it can be inferred that GRP94-substrate interactions would also be regulated, so that the lifetimes of GRP94-substrate interactions would be of a time frame relevant to protein synthesis and/or protein secretion. To achieve such regulation, we postulate that other as yet unidentified ER components physically interact with GRP94 to enhance the kinetics of substrate binding and/or release. Such factors, we hypothesize, might recognize specific conformations of GRP94, the bound substrate, or both. Should such regulatory factors be compartmentalized within the ER, the interaction of GRP94 with its substrates could be regulated at unique sites, such as the transitional elements-export sites (35, 36).

Because of the high degree of sequence homology (37), it is expected that members of the hsp90 family share a common...
molecular mechanism of action. Recently, it has been debated whether hsp90 proteins function in an ATP-dependent manner (38). In agreement with this study, it has been recently reported that cytosolic hsp90 does not bind ATP (38), does not contain bound nucleotides (39), and does not possess an ATPase activity (4, 38). Together, these findings provide a strong argument that hsp90 proteins function by an ATP-independent mechanism and will serve to direct future studies on the molecular regulation of hsp90 protein function.

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