

Adenosine Nucleotides and the Regulation of GRP94–Client Protein Interactions[†]

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Received March 8, 2004; Revised Manuscript Received April 27, 2004

ABSTRACT: The molecular chaperone heat shock protein 90 (Hsp90) serves essential roles in the regulation of signaling protein function, trafficking, and turnover. Hsp90 function is intimately linked to intrinsic ATP binding and hydrolysis activities, the latter of which is under the regulatory control of accessory factors. Glucose-regulated protein of 94 kDa (GRP94), the endoplasmic reticulum Hsp90, is highly homologous to cytosolic Hsp90. However, neither accessory factors nor adenosine nucleotides have been clearly implicated in the regulation of GRP94–client protein interactions. In the current study, the structural and regulatory consequences of adenosine nucleotide binding to GRP94 were investigated. We report that apo-GRP94 undergoes a time- and temperature-dependent tertiary conformational change that exposes a site(s) of protein–protein interaction; ATP, ADP, and radicicol markedly suppress this conformational change. In concert with these findings, ATP and ADP act identically to suppress GRP94 homooligomerization, as well as both local and global conformational activity. To identify a role(s) for ATP or ADP in the regulation of GRP94–client protein interactions, immunoglobulin (Ig) heavy chain folding intermediates containing bound GRP94 and immunoglobulin binding protein (BiP) were isolated from myeloma cells, and the effects of adenosine nucleotides on chaperone–Ig heavy chain interactions were examined. Whereas ATP elicited efficient release of BiP from both wild-type and mutant Ig heavy chain intermediates, GRP94 remained in stable association with Ig heavy chains in the presence of ATP or ADP. On the basis of these data, we propose that structural maturation of the client protein substrate, rather than ATP binding or hydrolysis, serves as the primary signal for dissociation of GRP94–client protein complexes.

GRP94,¹ the endoplasmic reticulum (ER) Hsp90 paralog, serves an essential role in the folding and secretion of a subset of membrane and secreted proteins such as Toll-like receptors 2 and 4, a subset of integrins, immunoglobulins, and thyroglobulin (1–7). Though multiple client protein substrates have been identified for GRP94, insights into the sequence or structural motifs recognized by GRP94 are sparse. In addition, the regulatory inputs governing GRP94–client protein interactions have not been established. GRP94 is highly homologous to Hsp90, so it is reasonable to expect that the two paralogs would display similar mechanisms of regulation. By way of analogy, cytosolic Hsp70 and its ER paralog, GRP78/BiP, display similar adenosine nucleotide-modulated interactions with client proteins, though they do differ in their selection of client protein substrates (8–11).

Hsp90 binds a multitude of cytosolic proteins, the majority of which serve cell-signaling functions (12–14). The ATPase activity of Hsp90 is known to serve a primary role in the

regulation of client protein binding and release with co-chaperones and accessory proteins providing key regulatory inputs to the central ATPase activity (13–18). In current models, preferred substrates for Hsp90 initially reside in complex with Hsp40/Hsp70 and are transferred to Hsp90 in a HSP organizing protein (Hop)-dependent reaction yielding Hsp40/Hsp70 release (14, 15, 17). The Hsp90–client protein complex then undergoes a process of “redecorating”, wherein p23 associates with the Hsp90 N-termini and tetratricopeptide (TPR) motif-bearing immunophilins associate with the C-terminus (14, 15, 17). The subsequent steps in the Hsp90 regulatory cycle are under continuing investigation, though it appears that conformational rearrangements in the Hsp90 N-termini serve as the rate-limiting step in a cycle of chaperone function with ATP hydrolysis eliciting client protein release. These latter stages are under the regulatory influence of Aha1 (activator of Hsp90 ATPase) (18–20).

A role for ATP in the regulation of Hsp90 activity was considered controversial for some time. However, pivotal crystallographic studies identifying an ATP/ADP binding pocket in the Hsp90 N-terminus clearly indicated a functional basis for adenosine nucleotide regulation of Hsp90 function (14, 21–24). In these studies, Hsp90 was shown to bind ATP in an unusual bent conformation via a structural domain referred to as the Bergerat fold (22, 25, 26). This motif is seen in a small, diverse array of proteins, the GHKL family, which includes bacterial DNA gyrase, bacterial histidine kinases, and MutL, a protein functioning in DNA repair (22, 25, 26). Though these proteins share the Bergerat fold and the capacity to bind ATP, they differ in utilizing either

[†] These studies were supported by a grant from the NIH (Grant DK47897 to C.V.N.) and a sponsored research agreement with Conforma Therapeutics (San Diego, CA).

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¹ Abbreviations: GRP94, glucose-regulated protein of 94 kDa; Ig, immunoglobulin; ANS, anilino-naphthalene-8-sulfonate; NECA, *N*-ethylcarboxamidoadenosine; Hsp90, heat shock protein 90; BiP, immunoglobulin binding protein; Hop, Hsp organizing protein.

ATPase or ATP phosphotransferase activities in their catalytic cycles. The N-terminal nucleotide-binding domain of Hsp90 was also cocrystallized with the fungal metabolites geldanamycin and radicicol (27–29). Both compounds function as adenosine nucleotide mimetics and target the Hsp90 N-terminal nucleotide-binding domain (27–29). Interestingly, these compounds do not bind to the nucleotide-binding domains of other GHKL family members. Geldanamycin binding has been shown to block the maturation of numerous Hsp90 substrates through induction of premature client protein release (30–32). Though ATP, ADP, and geldanamycin are known to differentially affect Hsp90–client protein interactions, the N-terminal ligand crystal structures of the ATP-, ADP-, geldanamycin-, and radicicol-bound forms are identical (16, 19, 20).

Though the sequence similarities between GRP94 and Hsp90 are very high, it is not clear that the two proteins display similar modes of regulation. Importantly, GRP94 lacks the C-terminal KEEVD domain, necessary for recognition by TPR motif-bearing accessory proteins. In addition and though there are ER-resident Hsp70 and Hsp40 (DnaJ) paralogs, no ER paralog of p23 has yet been reported. Like Hsp90, GRP94 can interact specifically with adenosine nucleotides (33). However, such interactions are of very low affinity and therefore undetectable by direct biochemical analysis (33, 34). For this very reason, we previously reported that GRP94 does not function as an ATP binding protein (33, 34). In subsequent studies, competitive displacement assays carried out with radiolabeled *N*-ethylcarboxamido-adenosine (NECA), an adenosine derivative known to bind GRP94 but not Hsp90, indicated that GRP94 displayed broad adenosine nucleotide/nucleoside recognition activity with binding activity observed for ATP, ADP, AMP, cAMP and adenosine (33). Genetic studies have demonstrated that point mutations in the nucleotide-binding pocket of GRP94 that prevent productive interactions with the adenine base block GRP94 function, so it appears that GRP94 function is linked to adenosine nucleotide binding (4, 35). However, it is not yet known whether ATP binding, and more critically, ATPase activity, is coupled to the cycle of GRP94–client protein recognition and release. Notably, GRP94 has an extremely low, NECA-insensitive ATPase activity (33). Second, and perhaps more significantly, the crystal structure of the NECA-bound form of the GRP94 N-terminal domain differs significantly from that of all available Hsp90 structures (29). In particular, all GRP94s contain a 3–5 amino acid insertion in the helix 1–4–5 subdomain that alters the conformation and ligand selectivity of the GRP94 N-terminal ligand binding pocket (29).

The identification of a conserved structural divergence between the N-terminal regulatory domains of GRP94 and Hsp90, coupled with the inability to identify an ATPase activity emanating from this domain, has prompted us to further evaluate the role of adenosine nucleotides in the regulation of GRP94–client protein interactions. In this study, we report that GRP94 undergoes a spontaneous, time- and temperature-dependent tertiary conformational change that exposes a protein–protein interaction site(s). The binding of adenosine ligands to the N-terminal regulatory domain suppresses this conformational change, thereby stabilizing the “closed-state” of GRP94. Differential scanning calorimetric analyses of ligand-induced structural stabilization

suggest that the adenine base, ribose, and α -phosphate moieties provide the critical binding interactions; the transitional unfolding temperatures of the Mg-ATP- and Mg-ADP-bound forms of GRP94 were essentially identical. To address the role of ATP and ADP in the regulation of GRP94–client protein interactions, Ig heavy chain–GRP94/BiP complexes were isolated from myeloma cell lines and the effects of adenosine nucleotides on chaperone–client protein interactions were assayed. Whereas addition of ATP elicited efficient BiP release from both wild-type and mutant Ig heavy chain intermediates, GRP94–Ig heavy chain complexes remained stable in the presence of ATP, ADP, or geldanamycin. On the basis of these data, we suggest that alterations in client protein conformation or assembly state, rather than ATP binding or hydrolysis, provide the critical signal in the GRP94–client protein dissociation reaction. In this view, adenosine nucleotides serve a global function in the regulation of GRP94 activity via suppression of GRP94 conformational dynamics.

EXPERIMENTAL PROCEDURES

Protein Purification. GRP94 was purified from porcine or canine pancreas as previously described (36). Briefly, pancreas tissue was processed by the procedure of Walter and Blobel to obtain a highly enriched rough microsome fraction (RM) (37). RMs were detergent-permeabilized by addition of 10 mM CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate) and subsequently centrifuged to yield a pellet fraction, containing endoplasmic reticulum membrane proteins, and a supernatant fraction, containing ER luminal proteins. GRP94 was purified from the ER luminal protein fraction by sequential anion exchange (SourceQ, AmershamPharmaciaBiotech, Piscataway, NJ) and gel filtration (Superdex 200, AmershamPharmaciaBiotech, Piscataway, NJ) chromatography. The GRP94-containing fractions obtained in the gel filtration step were pooled and concentrated by centrifugal ultrafiltration (Centricon-30; Amicon, Beverly, MA). Protein purity was routinely >95%, as assayed by Coomassie Blue staining of SDS–PAGE gels.

Reagents. Rabbit polyclonal antisera to GRP94 and BiP were prepared by contract service (Cocalico Biologicals, Reamstown, PA) and have been previously described (36). All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise noted. Geldanamycin was the kind gift of Dr. Len Neckers (National Cancer Institute, Rockville, MD).

Assays of Protein Conformation and Thermal Stability. I. Fluorescence Spectroscopy. Temperature and adenosine nucleotide/nucleoside ligand effects on GRP94 conformation were assayed with the environment-sensitive fluorophore anilinonaphthalene-8-sulfonate (ANS; Molecular Probes, Portland, OR). All experiments were conducted in a physiological salts buffer (PSB) consisting of 110 mM KOAc, 20 mM NaCl, 25 mM K-Hepes, pH 7.2, 2 mM Mg(OAc)₂, and 0.1 mM CaCl₂. Incubations were performed in a volume of 200 μ L with GRP94 present at a final concentration of 0.5 μ M. Ligands were added from 100 \times stocks with paired solvent controls being performed in parallel. Incubations were conducted in a thermostated water bath. At the termination of each incubation, ANS was added to a final concentration of 5 μ M, and following a 5 min incubation, fluorescence

spectra were collected (excitation 375 nm; emission 410–610 nm; 1 nm slit width) using a Fluoromax spectrofluorometer (Spex Industries, Inc., Edison, NJ).

II. Differential Scanning Calorimetry. Calorimetric analyses of ligand effects on GRP94 thermostability were performed on a VP-DSC differential scanning calorimeter (MicroCal Inc., Northampton, MA). GRP94 (1 mg/mL; 5.4 μ M) in physiological salts buffer was combined with the indicated ligands (diluted from 100 \times stocks) prior to analysis. The reference cell contained physiological salts buffer and ligand solvent at equivalent dilution. All samples were extensively degassed prior to analysis. Data were analyzed using the Origin software provided by the manufacturer.

III. Nondenaturing Polyacrylamide Gel Electrophoresis. GRP94 (0.3 mg/mL) was incubated in a physiological salts buffer in the presence or absence of ligands, as indicated. Samples were adjusted to 15% glycerol and subsequently loaded onto polyacrylamide gels lacking SDS (3% stacking gel; 6% separating gel). Separations were performed as described in ref 38.

Microsomal Chaperone Complex Formation. Chaperone–chaperone interactions occurring in the environment of the endoplasmic reticulum (ER) lumen were examined in ER-derived microsomes (RM), prepared as noted above. RMs were diluted in a RM dilution buffer (0.25 M sucrose, 50 mM KOAc, 5 mM Mg(OAc)₂) to a concentration of 10A₂₈₀ units/mL and incubated at the indicated temperature for 2 h in the presence or absence of GRP94-directed ligands. RMs were then detergent-permeabilized by addition of 10 mM CHAPS for 1 h on ice. The permeabilized RMs were centrifuged through a 0.5 M sucrose cushion (0.5 M sucrose, 25 mM K-Hepes, pH 7.2, 1 mM Mg(OAc)₂) at 50 000 rpm for 5 min (Beckman TLA100.2 rotor). Supernatant fractions, containing the soluble luminal proteins, were collected, and the pellet fractions, containing integral membrane proteins and large protein–protein complexes, were resuspended in SDS–PAGE sample buffer. Samples were separated on 12.5% SDS–PAGE gels and transferred to nitrocellulose for immunoblot analysis.

Ig Heavy Chain–Chaperone Interactions. SP2/0 M241 (mutant) and SP2/0 T15 WT H/L (wild-type) myeloma cells (17–19) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) containing *l*-glutamine, nonessential amino acids, sodium pyruvate, and 15% fetal calf serum. For analysis of Ig heavy chain–chaperone interactions, cells were lysed in ice-cold phosphate-buffered saline (PBS) containing 0.1% NP-40 and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were cleared by centrifugation at 10 000 rpm for 10 min. To capture Ig heavy chain–chaperone complexes, 50 μ L of a 50% slurry of protein A–agarose beads were added to the supernatant fractions and incubated for 90 min at 4 °C. In experiments where mutant Ig heavy chain–chaperone interactions were investigated, protein A beads were collected by centrifugation, washed with cell lysis buffer, and resuspended in a physiological salts buffer (110 mM KOAc, 20 mM NaCl, 5 mM Mg(OAc)₂, 0.1 mM CaCl₂, 25 mM K-Hepes, pH 7.2) in the presence or absence of the indicated ligands. The samples were incubated at room temperature for 30 min, and the beads were then collected by centrifugation. Beads were then washed in lysis buffer, and bound proteins were eluted with SDS–PAGE sample

buffer. With the SP2/0 T15 WT H/L cell lysates, GRP94-directed N-terminal ligands and a physiological salts buffer were included in the initial isolation step with the protein A beads. Beads were collected by centrifugation, washed, and eluted with SDS–PAGE sample buffer. Samples were run on 12.5% SDS–PAGE gels and transferred to nitrocellulose for immunoblot analysis.

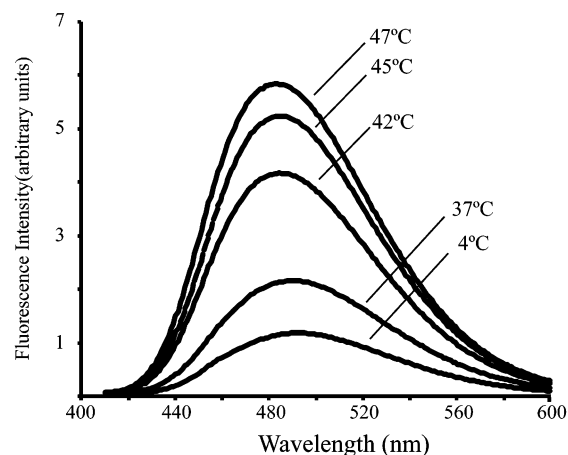
RESULTS

Hsp90–client protein interactions are regulated via cycles of ATP binding and hydrolysis (13, 14, 17). Though well-established for Hsp90, a role for ATP binding and hydrolysis in the regulation of GRP94–client protein interactions has not been identified to date. To gain insight into the functional consequences of ATP binding to GRP94, we have examined the role of ATP in the regulation of GRP94 structure, conformation, and client protein interactions.

Ligand interactions with proteins are accompanied by changes in protein structure and conformational dynamics (39–41). In past investigations into GRP94 conformational dynamics, it was reported that GRP94 undergoes an irreversible tertiary conformational change following exposure to elevated (>37 °C) temperatures (38, 42). Significantly, under these conditions, no change in GRP94 secondary structure was observed (38, 42). This conformational change is accompanied by enhanced molecular chaperone activity, as measured by *in vitro* client protein aggregation assays, homooligomerization, and enhanced binding of the environment-sensitive fluorophore ANS (33, 38, 42). We used the ANS binding assay to characterize the temperature and time dependence of the GRP94 temperature-induced conformational change (Figure 1A,B). As depicted in Figure 1A, GRP94 readily displays a thermally driven tertiary conformational change with enhanced binding of ANS occurring following incubation of apo-GRP94 (ligand-empty) at 37 °C and more dramatically at elevated (42–47 °C) temperatures. The observed conformation change is time-dependent; incubation of apo-GRP94 for extended time periods at 37 °C allows the slow conformational conversion of GRP94 to the “open”, ANS-binding conformation (Figure 1B).

Ligand binding to proteins commonly results in conformational stabilization, due to the coupling of binding and unfolding equilibria (39, 43, 44). To assess the role of ligand binding to the N-terminal GRP94 adenosine nucleotide-binding domain on the described conformational conversion, GRP94 solutions were incubated with increasing concentrations of ligands and subsequently incubated at 47 °C. As shown in Figure 2, panels A–C, the addition of increasing concentrations of either ATP, *N*-ethylcarboxamidoadenosine (NECA), a GRP94-specific N-terminal ligand (33, 45), or radicicol resulted in a dose-dependent inhibition of the tertiary conformational conversion. Maximal suppression of GRP94 conformational dynamics required that ligands be present at saturating concentrations; which, as indicated, occurred at 1–5 mM ATP, 0.3–1 mM NECA, or 0.3–3 μ M radicicol (Figure 2A–C). The data regarding ATP-mediated suppression of GRP94 conformational dynamics are of particular interest. Previously, we had reported that GRP94 did not display ATP binding activity, as determined by standard ligand binding techniques (34). The data in Figure 2A clearly indicate that ATP can associate with GRP94 with a half-maximal suppression of the assayed

A.



B.

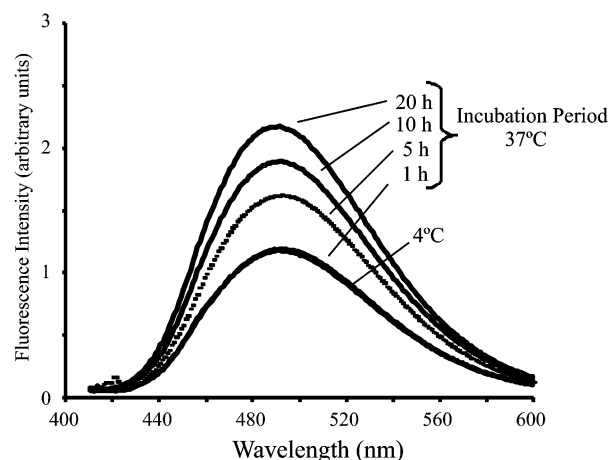


FIGURE 1: GRP94 undergoes a time- and temperature-dependent tertiary conformational change. Native GRP94 (0.5 μ M) was incubated in a physiological salts buffer at the indicated temperatures for 2 h (A) or at 37 $^{\circ}$ C for the indicated times (B). After the incubation, the fluorescent dye, ANS, was added to a final concentration of 5 μ M, and fluorescence emission spectra were recorded from 410–610 nm (excitation 375 nm).

tertiary conformational conversion occurring at ATP concentrations of ca. 300–400 μ M. The assay systems used previously are unable to detect such weak interactions; we thus revise these past conclusions to indicate that GRP94 is capable of low-affinity interactions with ATP. For all ligands assayed, saturating ligand concentrations did not completely block the heat-induced conformational conversion, suggesting the existence of multiple ANS binding sites, perhaps differing in their coupling to the N-terminal adenosine nucleotide-binding domain. In summary, the data depicted in Figures 1 and 2 indicate that in the absence of N-terminal ligands, GRP94 undergoes a time- and temperature-dependent conformational conversion to assume a conformer state displaying enhanced ANS binding. In the presence of N-terminal ligands, this conformational conversion is suppressed. Interestingly, ligands thought to act as inhibitors of GRP94 function (radicol) and ligands thought to regulate the conformational interconversions that accompany client protein binding and release (ATP) act identically to suppress GRP94 conformational dynamics.

In a previous study we reported that the GRP94 N-terminal ligand-binding pocket accommodated a broad array of

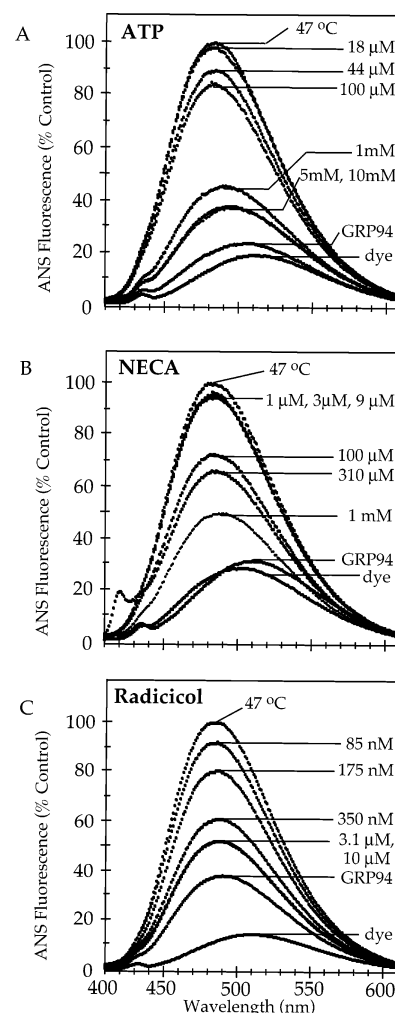


FIGURE 2: N-terminal-directed ligands suppress GRP94 conformational transitions. Native GRP94 (0.5 μ M) was incubated with the indicated concentrations of ligands at 47 $^{\circ}$ C for 1 h. The fluorescent dye, ANS, was then added to a final concentration of 5 μ M, and emission spectra were collected from 410–610 nm (excitation 375 nm). For comparison, the ANS emission spectrum of a 0.5 μ M solution of GRP94 prior to heat treatment is included (GRP94).

adenosine ligands, with significant binding interactions observed with ATP, ADP, AMP, cAMP, NECA, and free adenosine (33). This binding profile reflects the stringent base selection in the conserved adenosine-binding pocket and the diversity of potential binding interactions available for the ribose and ribose-substituent moieties (22, 29). As previously noted, ligand binding influences protein thermal stability by shifting the equilibrium between folded and unfolded conformers (40). Thus, both ligand binding chemistry and ligand occupancy state can contribute to ligand-mediated suppression of thermal unfolding. To examine the contribution of the ribose 5' substituents to the thermally driven GRP94 tertiary conformational change, GRP94 was incubated in either the absence of ligand or the presence of either half-maximal or saturating concentrations of adenosine (5' = hydroxyl), NECA (5' = ethylcarboxamido), or ATP (5' = α,β,γ phosphate ester), and the conformational response to 47 $^{\circ}$ C treatment was determined. As shown in Figure 3, maximal suppression of the tertiary conformational change is observed in the presence of saturating concentrations of ligand. The capacity to assume the "open state" conformation

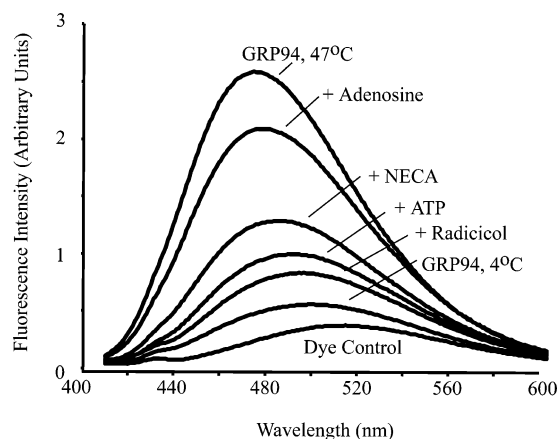


FIGURE 3: GRP94 N-terminal ligands suppress GRP94 conformational dynamics. Maximal ligand occupancy concentrations were determined by NECA competition assays conducted at 37 °C. GRP94 (0.5 μ M) was then incubated with the saturating concentrations of the indicated ligands for 1 h at 47 °C. At the completion of the incubation, ANS was added to a final concentration of 5 μ M, and emission spectra (410–610 nm; excitation 375 nm) were collected.

was strongly influenced by the 5' substituent chemistry with adenosine serving as a relatively poor stabilizing ligand and NECA and ATP mirroring, at saturating concentrations, the potent pan-Hsp90 inhibitor radicicol (Figure 3). These data suggest that GRP94 may exist in different conformational states when bound to the different ligands. This conclusion is supported by previous data demonstrating distinct tryptophan fluorescence spectra for GRP94 in complex with different N-terminal ligands (33). As well, ligand modeling studies of the GRP94 N-terminal domain–NECA complex identifies a steric clash between the α and β phosphate of ATP and the side chain of GRP94 residue G196, indicating that different conformational states are likely required to accommodate NECA and ATP (29).

The ANS binding assay serves as a sensitive measure of local conformational changes. To investigate the consequences of ligand binding on the global thermal stability of GRP94, differential scanning calorimetry (DSC) of GRP94–ligand complexes was performed. Thermograms of ligand-free GRP94, GRP94/Mg²⁺, GRP94/Mg-ATP, GRP94/Mg-ADP, GRP94/NECA, and GRP94/radicicol complexes are depicted in Figure 4A–C. A temperature scan of native, ligand-free GRP94 shows an exothermic transition centered at 51.3 °C (Figure 4A). If GRP94 is pretreated at 50 °C for 15 min, the thermogram shows a very small exothermic transition indicating that the conformational change is irreversible (Figure 4A). Inclusion of 10 mM Mg-ATP or 10 mM Mg-ADP with the GRP94 results in a shift in the transition temperature to 54.1 and 54.4 °C, respectively. It can be concluded from these data that any binding interactions occurring via the γ phosphate do not contribute significantly to the global ligand stabilization of GRP94 structure (Figure 4B). Interestingly, in the available atomic structures of Hsp90–adenosine nucleotide complexes, no direct or indirect binding interactions between the γ phosphate and Hsp90 amino acid side chains are discernible (22). Radicicol, which was shown to be the most effective ligand in inhibiting the conformational transition in the ANS fluorescence assay, is also the most effective ligand in shifting the global thermal transition, yielding a midpoint

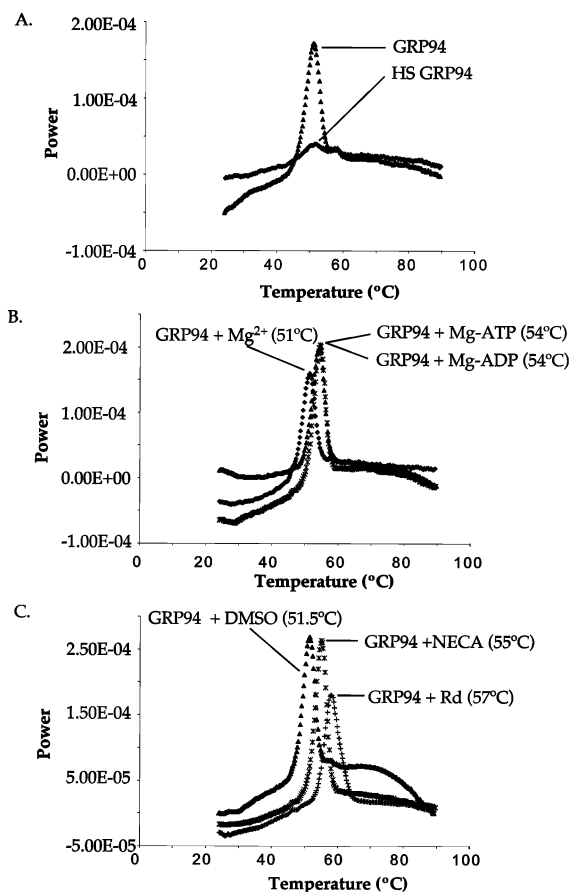


FIGURE 4: N-terminal ligands enhance the global thermal stability of GRP94. GRP94 (5.4 μ M) was incubated in physiological salts buffer and subjected to a thermal scan from 20 to 90 °C in a differential scanning calorimeter (DSC). In panel A, in the absence of ligand, GRP94 exhibits an exothermic conformational transition, centered at 51.3 °C. Preincubation of GRP94 at 50 °C for 15 min prior to scanning abolishes the observed signal. In panel B, addition of 10 mM Mg-ATP or 10 mM Mg-ADP to solutions of GRP94 results in a shift in the conformational transition temperature to 54.1 or 54.4 °C, respectively. In panel C, addition of 0.5 mM NECA or 50 μ M radicicol shifts the GRP94 conformational transition temperature to 55 or 57.3 °C, respectively. The radicicol solvent (1% DMSO) only modestly alters the transition temperature (increase of 0.2 °C).

temperature of 57.3 °C. NECA, which was modestly less effective than ATP in suppressing the tertiary conformational change assayed by ANS (Figure 3) was more effective in the global stabilization of GRP94 structure with a midpoint transition of 55 °C. This difference further supports the conclusion that the NECA-bound conformation of GRP94 differs from the ATP-bound state (33). The above data indicate that ADP and ATP elicit similar, perhaps identical, conformer states of GRP94.

Recent crystallographic analyses of both the Hsp90 and GRP94 N-terminal domains have identified a protein–protein interaction site that, in the case of Hsp90, functions in the N-terminal dimerization reaction that accompanies ATP binding (46, 47). Interestingly, the tertiary conformational change that occurs upon incubation in the absence of adenosine nucleotides (Figures 1 and 2) is accompanied by GRP94 homooligomerization (Figure 5A). Identical results were obtained with a recombinant amino-terminal domain encompassing residues 69–337, thereby identifying the amino-terminal domain as a site for conformational-change-

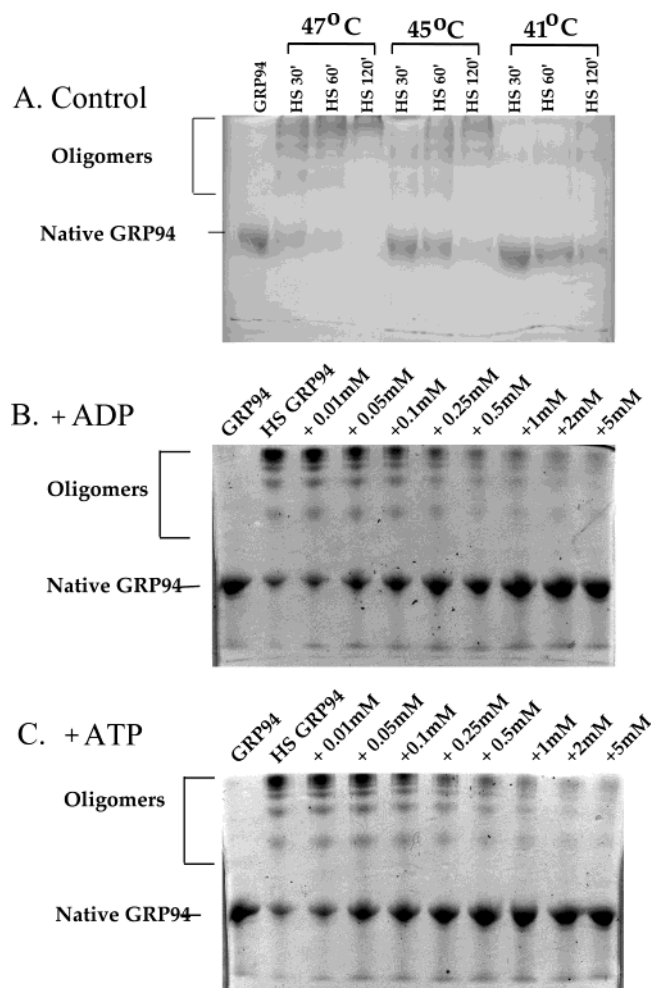


FIGURE 5: GRP94 undergoes a time- and temperature-dependent homooligomerization reaction. GRP94 (0.3 mg/mL) was incubated in the physiological salts buffer at (A) the indicated temperature for either 30, 60, or 120 min. In panels B and C, ADP or ATP was added to the GRP94 at the indicated concentrations, and the mixture was incubated at 47 °C for 1 h. Samples were fractionated on native gels and visualized by Coomassie Blue staining. The positions of the predominant native and oligomeric forms of GRP94 are indicated to the left of the digital gel image.

driven GRP94 oligomerization (data not shown). As shown in Figure 5A, the propensity for GRP94 oligomer formation was proportional to the duration and temperature of the incubation, as reported previously for Hsp90 (48). Significantly, exposure of this protein–protein interaction site was regulated by adenosine nucleotides, ATP and ADP displaying near identical efficacies in suppressing the heat-induced homooligomerization (Figure 5B,C). These results mirror those presented in Figures 1–4 and reiterate a common structural theme; the range of conformers accessed by GRP94 include structural forms capable of enhanced protein–protein interaction. Adenosine nucleotides, in general, alter the equilibria between these conformer states and favor a conformer displaying low conformational mobility and protein interaction capacity.

The experiments described in Figure 5 were conducted with relatively dilute solutions of purified native GRP94. In the endoplasmic reticulum, however, GRP94 resides in a crowded environment containing high concentrations of other ER-resident chaperones, such as BiP, ERp72, PDI, and calreticulin. To examine whether such homooligomerization

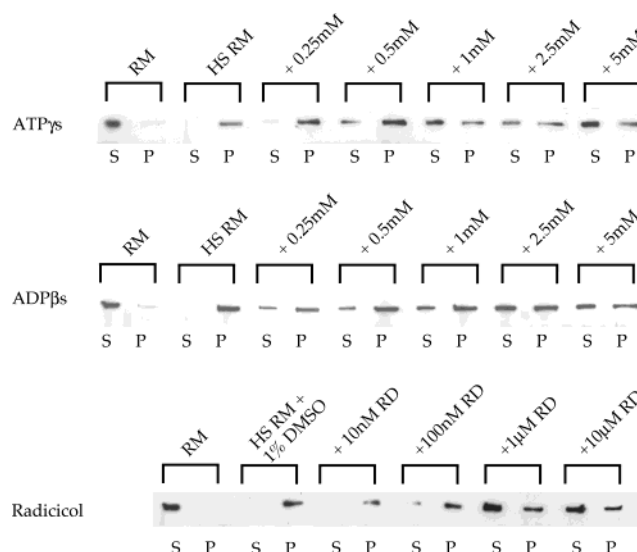


FIGURE 6: N-terminal ligands inhibit the ability of GRP94 to form high molecular weight oligomers in an ER luminal environment. ER-derived microsomes (RMs) were incubated for 2 h at 4 or at 42 °C in the presence or absence of ligands. RMs were then permeabilized by addition of 10 mM CHAPS and centrifuged through a 0.5 M sucrose cushion at 50 000 rpm for 5 min in the Beckman TL100.2 rotor. Supernatant and pellet fractions were collected and run on a 12.5% SDS–PAGE gel. The presence of GRP94 in the supernatant or pellet fraction was determined by immunoblot analysis.

activity was displayed in a more native environment, ER-derived microsomes were incubated in the presence or absence of GRP94-directed ligands (ATP γ S, ADP β S, radicicol) and briefly exposed to elevated temperatures, and the formation of higher order oligomers was assayed. The results of these experiments are depicted in Figure 6. As seen with the purified native protein, microsomal GRP94 efficiently entered higher order oligomers and so was recovered in the pellet fraction after centrifugation, following exposure to elevated temperatures. Also as seen with the purified native protein, these observed changes in quaternary structure were markedly suppressed in the presence of adenosine nucleotides or radicicol. It should be noted that in the experiments depicted in Figure 6, nonhydrolyzable forms of ATP and ADP were used because control experiments demonstrated that microsomal vesicles display substantial ATPase and ADPase activities (data not shown). To determine the molecular composition of the microsome-derived GRP94-oligomer fraction, the pellet fraction was recovered and analyzed by SDS–PAGE. A Coomassie Blue image of a representative gel is depicted in Figure 7A and demonstrates that the oligomer fraction contains a subset of the total ER luminal protein contents and is notably absent in detectable levels of calreticulin and PDI, two abundant components of pancreatic rough microsomes (relative mobility of calreticulin and PDI indicated by the asterisk). To evaluate whether microsome-associated GRP94 assembled into hetero-oligomeric complexes, microsomal lumen contents derived from control microsomes and microsomes treated at elevated temperatures were released by detergent treatment, and the GRP94 fraction was selected by Concanavalin A–Sephadex isolation. As shown in Figure 7B, control luminal protein fractionations conducted in the absence of Concanavalin A–Sephadex yielded the expected recovery of GRP94 in the supernatant fraction, whereas in the presence of Con-

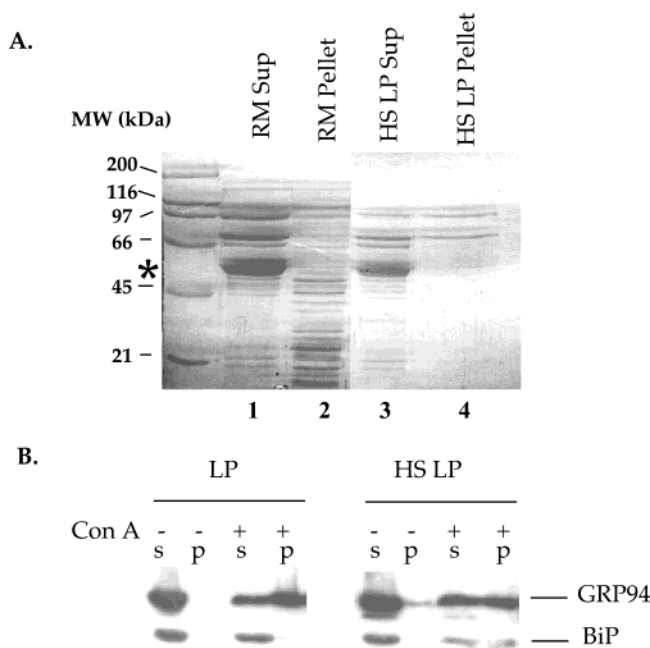


FIGURE 7: In response to elevated temperatures, GRP94 associates with ER luminal chaperones to form high molecular weight complexes. In panel A, ER-derived microsomes (RMs) were permeabilized by addition of 10 mM CHAPS, and the ER luminal proteins were separated from the ER membranes by centrifugation. The protein composition of the pellet (ER membrane; lane 1) and supernatant (ER luminal proteins; lane 2) fractions was determined by SDS-PAGE. A digital image of a Coomassie Blue stained gel is depicted. The supernatant fraction, containing the ER luminal proteins, was treated at 42 °C for 2 h and centrifuged at 50 000 rpm for 30 min, and the supernatant (HS supernatant) and pellet (HS pellet) fractions were collected. The protein composition of each fraction was determined by SDS-PAGE and Coomassie Blue staining (lanes 3 and 4). The asterisk indicates the region of the gel containing calreticulin and PDI. In panel B, RMs were diluted in a glycerol buffer (10% glycerol, 50 mM KOAc, 5 mM Mg(OAc)₂), and ER luminal proteins were isolated by addition of 10 mM CHAPS and centrifugation through a glycerol cushion at 50 000 rpm for 30 min. Luminal proteins (LP) were then incubated at either 4 °C (LP) or 42 °C (HS LP) for 2 h. Concanavalin-A Beads were added where indicated, and following incubation, the samples were then centrifuged briefly to isolate a supernatant fraction (containing the nonglycoprotein components) and pellet fraction (containing the resin-associated glycoproteins and any associated nonglycoproteins). Immunoblots for GRP94 or BiP were then performed on the supernatant and pellet fractions.

canavalin A-Sepharose, GRP94, but not BiP, was recovered in association with the resin (pellet) fraction. In contrast, when similar fractionations were conducted on luminal contents derived from heat-treated microsomes, BiP was recovered in the Concanavalin A-bound fraction, consistent with it entering a hetero-oligomeric complex with GRP94. These results are in agreement with recent data demonstrating that GRP94 and BiP can enter hetero-oligomeric complexes under native conditions (49).

The preceding data, examining the structural consequences of adenosine nucleotide binding to GRP94, suggest that ATP and ADP can serve similar roles in suppressing the conformational mobility of GRP94. The ATP- and ADP-bound forms of GRP94 may thus represent identical conformer states. Alternatively, the ATP- and ADP-bound forms of GRP94 may exist in similar but distinct conformer states, possibly differing in functional properties. Should the ATP- and ADP-bound forms differ in their interaction with client

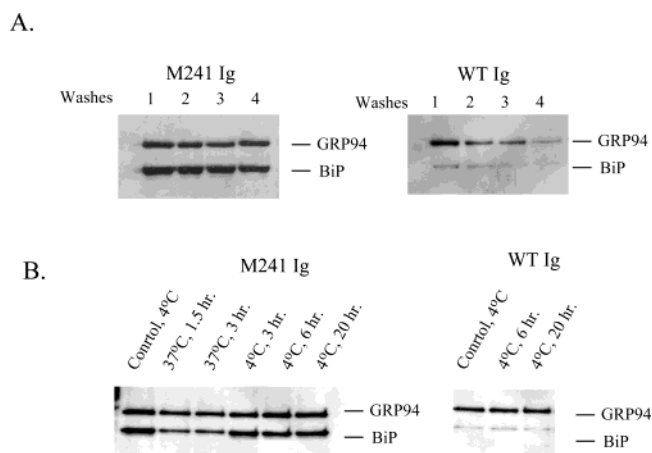


FIGURE 8: GRP94 and BiP are found in association with both mutant and wild-type Ig heavy chains. SP2/0 M241 (mutant) and SP2/0 T15 WT H/L (wild-type) myeloma cells were lysed in ice-cold PBS containing 0.1% NP-40 and 1 mM PMSF and cleared by centrifugation at 10 000 rpm for 10 min. Ig complexes were then captured by addition of protein A-agarose beads. The presence of GRP94 and BiP was determined by immunoblot. In panel A, the indicated number of washes was conducted with the cell lysis buffer, and the stability of the complexes was determined by immunoblot analysis of GRP94 and BiP content. In panel B, isolated Ig heavy chain-chaperone complexes were incubated at 37 °C for the indicated time period, and complex stability was assayed by immunoblot analysis of GRP94 and BiP content.

proteins, as predicted from available data regarding Hsp90, the latter scenario would be favored. To distinguish between the two scenarios, the role of ATP and ADP in the regulation of GRP94-client protein interactions was examined. In these experiments, we utilized two SP2/0 myeloma cell lines expressing either a wild-type (SP2/0 T15) or a mutant Ig heavy chain (SP2/0 M241). SP2/0 T15 has been previously demonstrated to secrete Ig, whereas in the mutant cell line SP2/0 M241 Ig heavy chains are retained within the ER in complex with GRP94 and BiP (50). Ig heavy chain-chaperone complexes isolated from wild-type and mutant cells can be readily distinguished; whereas GRP94 and BiP association with the mutant Ig heavy chain is robust, the wild-type Ig heavy chain is recovered in association with relatively low levels of GRP94 and comparatively low levels of BiP (Figure 8A). Additionally, the mutant Ig-chaperone complexes were stable to repeated cycles of washing; such treatment resulted in the gradual dissociation of GRP94 and BiP from the WT Ig (Figure 8A). Once isolated, both wild-type and mutant chaperone Ig complexes are stable over time with the mutant Ig-chaperone complexes remaining in association following extended incubation at 4 or 37 °C (Figure 8B).

To assess the role of ATP binding/hydrolysis in the regulation of GRP94-client (Ig heavy chain) interactions, wild-type Ig-chaperone and mutant Ig-chaperone complexes were isolated and incubated in the presence of nucleotides, nucleotide/nucleotide regenerating systems, and known GRP94-directed ligands, and Ig-chaperone complex association states were analyzed (Figure 9A). The addition of ATP to the mutant Ig-chaperone complexes resulted in efficient release of BiP but not GRP94 (Figure 9A). Similarly, addition of ATP in a detergent-molybdate buffer known to accentuate ATP-dependent p23 association with Hsp90 or an ATP-regenerating system, elicited BiP but not

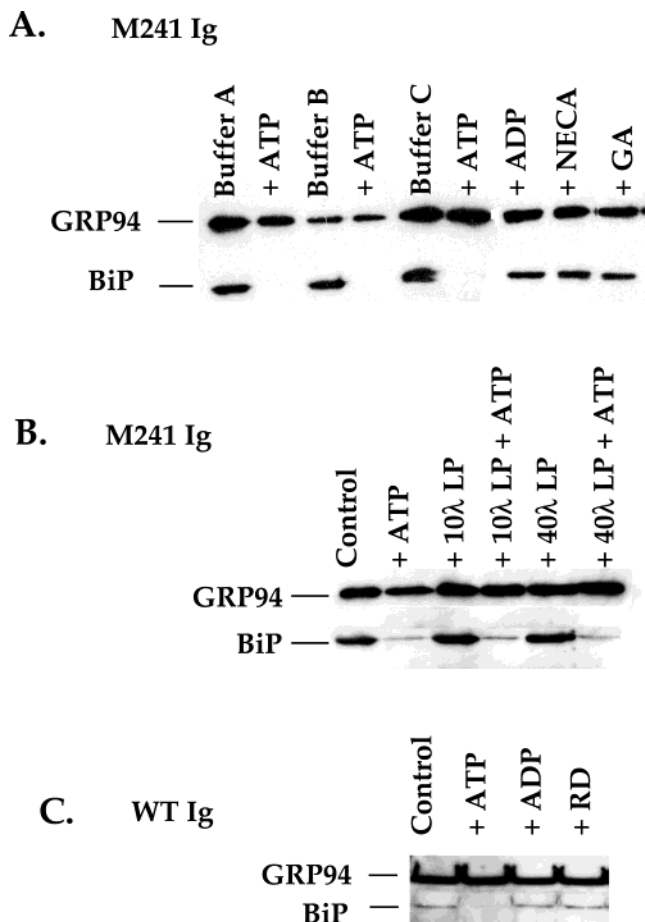


FIGURE 9: The addition of MgATP to isolated Ig-chaperone complexes elicits release of BiP but not GRP94. In panel A SP2/0 M241 cell lysates were prepared by lysis of cells in PBS plus 0.1% NP-40 and cleared by centrifugation at 10 000 rpm for 10 min. Protein A beads were then added, and samples were incubated at 4 °C for 90 min. Beads and associated proteins were isolated by centrifugation and resuspended in physiological salts buffer in the presence or absence of the indicated ligands (10 mM ATP, 10 mM ADP, 0.5 mM NECA, or 10 μ M geldanamycin). Where indicated, an ATP regenerating system or a molybdate buffer (54) was also included in this incubation. The lysates were then incubated at 25 °C for 30 min, and the protein A beads and associated proteins were reisolated by centrifugation. The presence of GRP94 or BiP was determined by immunoblot. Buffer A = control buffer (PBS); buffer B = molybdate/detergent buffer (54); buffer C = ATP regeneration system buffer. In panel B, ER luminal proteins were isolated from ER-derived microsomes by permeabilization with 10 mM CHAPS and concentration of the resulting supernatant fraction to the original microsome solution volume. Indicated amounts of luminal proteins (1 λ = 1 equiv of microsomes; ref 37) were added back to isolated Ig/GRP94/BiP complexes in the presence or absence of ATP. Samples were then analyzed as described in panel A. In panel C, SP2/0 T15 WT H/L cell lysates were prepared by incubation in PBS with 0.1% NP-40 and cleared by centrifugation at 10 000 rpm for 10 min. Cleared cell lysates were then supplemented with a 10 \times physiological salts buffer (final concentration of 110 mM KOAc, 20 mM NaCl, 5 mM Mg(OAc)₂, 0.1 mM CaCl₂, and 25 mM K-Hepes), the indicated ligands, and protein A agarose beads. Lysates were then incubated at 25 °C for 30 min. Protein A beads and associated proteins were isolated by centrifugation, and samples were analyzed for the presence of GRP94 and BiP by immunoblot.

GRP94 release (Figure 9A). ADP, NECA, and geldanamycin were without effect on both GRP94- and BiP-Ig interactions.

Regulated assembly/disassembly of Hsp90-client protein interactions occurs in the context of numerous cochaperones and accessory factors (14, 17). Though ER-resident paralogs of the extended Hsp90 machinery have not yet been identified, it is possible that the absence of ATP-elicited client protein from GRP94 requires additional components. To address this possibility, mutant Ig-chaperone complexes were supplemented with ATP and increasing concentrations of concentrated ER luminal contents, containing the entire repertoire of ER resident luminal components (Figure 9B). Under these conditions as well, the addition of ATP, in the presence or absence of luminal proteins, resulted in efficient release of BiP but no reduction of GRP94-Ig heavy chain interactions. The insensitivity of GRP94-Ig interactions to the presence of ATP was not unique to the mutant heavy chain component investigated; a similar, if not identical, pattern of ATP-elicited chaperone release was observed for the wild-type Ig heavy chain-chaperone complex as well (Figure 9C). As will be later discussed, such data suggest alternative mechanisms for the regulation of GRP94-client protein interactions.

DISCUSSION

Adenosine nucleotides serve key regulatory functions in the regulation of Hsp90-client protein interactions. In this report, we have examined the structural and functional consequences of GRP94-adenosine nucleotide interactions and report the following primary observations. One, ATP and ADP function identically to suppress GRP94 conformational dynamics. Two, in the environment of the endoplasmic reticulum, the nucleotide-empty-state form of GRP94 can assume a conformation that is accompanied by enhanced interactions with BiP. Three, neither ATP nor ADP elicits GRP94 dissociation from client protein complexes. The latter observation suggests that the primary mechanism of client protein release from GRP94 is independent of adenosine nucleotides.

In previous studies, we reported that GRP94 displays an unusually weak ATPase activity and specific, though very low-affinity (>0.5 mM K_d), interactions with adenosine nucleosides and nucleotides (33, 34). Significantly, the observed ATPase activity was insensitive to the presence of *N*-ethylcarboxamidoadenosine (NECA), an adenosine receptor agonist that displays relatively high equilibrium binding affinities (200 nM) for the N-terminal nucleotide-binding domain of GRP94, but not Hsp90 (33, 45). The inability to detect significant GRP94 ATPase activity, combined with the weak affinities of the GRP94 GHKL binding pocket for ATP and ADP, prompt questions regarding the biological role of adenosine nucleotides in the regulation of GRP94 (21, 33, 34). In particular, we were interested in determining whether the ATP and ADP binding elicited distinct conformer states of GRP94 and whether such postulated conformer-state differences would differ in their interactions with client protein substrates. The existence of such differences would support the hypothesis that GRP94-client protein interactions are regulated via nucleotide-binding reactions occurring in the N-terminal regulatory domain.

GRP94 undergoes a temperature- and time-dependent tertiary conformational change that is accompanied by enhanced molecular chaperone activity (38, 42). As seen with

Hsp90, this conformational change, referred to as the “open-state”, is accompanied by the exposure of a protein–protein interaction site(s) and the formation of discrete homooligomers. Using the environment-sensitive fluorophore ANS as a probe for local conformational changes, we observed that ligands that bind the N-terminal regulatory domain suppress the conformational conversion to the open state. In addition, both ATP and ADP were found to display identical activity in the suppression of GRP94 homooligomerization. Interestingly, in numerous assays of GRP94 conformational dynamics (ANS binding, tryptophan fluorescence, protease sensitivity, quaternary structure analyses), ATP and ADP have been found to behave similarly to established pan-inhibitors of Hsp90 function (geldanamycin and radicicol) in suppressing GRP94 conformational dynamics (21, 33, 38). Indeed, differential scanning thermal calorimetry indicated that the temperature midpoints for the primary exothermic conformational transition of the GRP94–ATP and GRP94–ADP complexes were identical (54 °C), suggesting that the two nucleotide ligands are engaged in similar, if not identical, binding interactions in the GRP94 nucleotide-binding pocket. This proposal is consistent with crystal structure data from Hsp90–ATP complexes, in which the γ -phosphate does not appear to be engaged in any direct or indirect binding interactions and the ATP- and ADP-ligated forms of the Hsp90 N-terminal region have essentially identical structures (17, 22). Such data do imply that ATP and ADP may perform a common function in the regulation of GRP94 activity.

The marked similarities observed in the conformational response of GRP94 to ATP or ADP binding may reflect an inability of the analytical methods to detect subtle but functionally relevant ATP- or ADP-specific conformer states. In principle, a stabilization of protein conformation is expected upon ligand binding, so it is reasonable to expect that the ATP- and ADP-bound forms of GRP94 could comprise similar but distinct conformer states with similar thermal stabilities (39, 41). Given the available biological precedent indicating distinct roles for ATP and ADP in the regulation of Hsp90–client protein interactions, the existence of distinct GRP94–ATP and GRP94–ADP conformer states would appear likely. The biological basis for this prediction was examined in analyses of the role(s) of ATP and in the regulation of GRP94–client protein interactions. For these experiments, GRP94–Ig complexes bearing either wild-type or mutant forms of Ig heavy chain were isolated, and the capacity of either ATP or ADP to elicit GRP94 release was determined. For both wild-type and mutant forms of the protein, neither ATP nor ADP elicited the dissociation of GRP94 from Ig. Interestingly, BiP, which was present in the complexes, was readily released following addition of ATP. Additional control experiments, in which the GRP94–Ig complexes were supplemented with total endoplasmic reticulum luminal proteins, provided no indication that the inability of ATP to elicit client protein release from GRP94 was due to limiting (putative) accessory or cochaperone proteins. In the absence of data demonstrating that ATP can elicit client protein release from GRP94, we suggest that dissociation of GRP94–client protein complexes is driven by client protein folding/assembly rather than ATP binding/hydrolysis. In this view, GRP94 functions as a “holdase” with release of the client protein accompanying its structural maturation, oligomeric assembly, or both.

Though GRP94 and Hsp90 differ in the cohort of known accessory factors and cochaperones that govern their interactions with client proteins, the high sequence and structural similarities between GRP94 and Hsp90 suggest similar, if not identical, modes of regulation. Recent biochemical and structural studies suggest, however, that conclusions regarding shared modes of regulation by GRP94 and Hsp90 should be evaluated with caution. For example, the two proteins can be readily distinguished by their capacity for binding of the substituted adenosine molecule NECA to the N-terminal nucleotide-binding site; whereas GRP94 binds 1 mol of NECA per GRP94 dimer, Hsp90 is refractory to NECA binding (33, 45). Such differences derive from a highly conserved structural divergence between the two proteins. As discussed in a recent study, all known GRP94s contain a 3–5 amino acid insertion in a region referred to as the helix 1–4–5 subdomain, which comprises an essential structural element of the nucleotide-binding pocket (29). This conserved insertion allows GRP94 to accommodate the 5' ethylcarboxamido substituent of NECA and may form an essential element of a conformational switch; modeling studies indicate that a substantial conformational change in the GRP94 1–4–5 subdomain is required for interactions with adenosine nucleotides (29).

A definitive role for ATP binding/hydrolysis in the regulation of GRP94–client protein interactions has so far proven elusive, so alternative models of adenosine nucleotide function in the regulation of GRP94 activity can be considered. For example, it is possible that adenosine nucleotides serve a “stress sensor” function in the regulation of GRP94 activity. In this model, decreasing ATP levels, as would accompany many forms of cell stress, would favor the ligand-empty form of GRP94, a scenario that is further favored by the very low equilibrium binding affinities of GRP94 for ATP and ADP. Under these conditions, the conformational repressor function of ATP would be relieved, so it would be predicted that GRP94 would be capable of additional or alternative protein–protein interactions. Such interactions could comprise both altered and extended interactions with client proteins or with neighboring chaperone proteins, such as BiP. Interestingly, stress conditions known to yield a decrease in cellular ATP levels elicit an increase in AMP-activated protein kinase activity (51). This family of highly conserved kinases, which includes the yeast SNF1 kinase, function in the response to environmental or nutritional stress via regulation of ATP-requiring catabolic pathways. In a related manner, the kinase mTOR (mammalian target of rapamycin) has recently been proposed to function as an energy sensor with decreases in ATP concentration yielding an activation of mTOR kinase activity (52). Given that many environmental stress conditions that yield a decrease in cellular ATP levels, such as glucose deprivation, also elicit the activation of the ER unfolded protein response, it is intriguing to consider that declining ATP levels serve as a signal for the activation (derepression) of GRP94 function. Because the affinities of Hsp90 for adenosine nucleotides are also quite weak, this proposal may also be relevant to models of Hsp90 function during cell stress and recovery, in particular, the provocative hypothesis that Hsp90 chaperones function as molecular capacitors for evolutionary change (53).

The cytosol and the ER lumen differ in the chemical environments in which protein folding is conducted and, as importantly, in the temporal constraints on chaperone–client protein interaction. Concerning the latter, client proteins reside only briefly in the ER lumen; following proper folding and oligomeric assembly, client proteins exit this specialized protein-folding environment to enter the secretory pathway. In contrast, cytosolic Hsp90–client protein complexes can be long-lived. With the glucocorticoid and progesterone receptors as prominent examples, assembly of a steroid receptor/multichaperone complex culminates in the formation of a temporally stable Hsp90–receptor complex that resides in the cytosol awaiting steroid binding and translocation to the nucleus (14). In light of such clear differences, that is, the formation of stable (Hsp90) vs transient (GRP94) client protein–chaperone complexes, it is reasonable to consider that the biological basis for the regulation of GRP94–client protein interactions differs from that of Hsp90.

ACKNOWLEDGMENT

The myeloma mutant cell lines SP2/0 M241 and SP2/0 T15 were the generous gift of Dr. Marvin Rittenberg, Oregon Health Sciences University, Portland, Oregon. The authors are grateful to Drs. Jeff Baker and Daniel Gewirth for critical comments and helpful discussions and to Dr. Ashutosh Chilkoti for the use of the differential scanning calorimeter.

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BI049539Q