Structure of Unliganded GRP94, the Endoplasmic Reticulum Hsp90

BASIS FOR NUCLEOTIDE-INDUCED CONFORMATIONAL CHANGE*

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GRP94, the endoplasmic reticulum paralog of Hsp90, is regulated by adenosine nucleotides that bind to its N-terminal regulatory domain. Because of its weak affinity for nucleotides, the functionally relevant transition in GRP94 is likely to be between the unliganded and nucleotide-bound states. We have determined the structure of the unliganded GRP94 N-domain. The helix 1-4-5 subdomain of the unliganded protein adopts the closed conformation seen in the structure of the protein in complex with inhibitors. This conformation is distinct from the open conformation of the subdomain seen when the protein is bound to ATP or ADP. ADP soaked into crystals of the unliganded protein reveals an intermediate conformation midway between the open and closed states and demonstrates that in GRP94 the transition between the open and closed states is driven by ligand binding. The direction of the observed movement in GRP94 shows that nucleotides act to open the subdomain elements rather than close them, which is contrary to the motion proposed for Hsp90. These observations support a model where ATP binding dictates the conformation of the N-domain and regulates its ability to form quaternary structural interactions.

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The atomic coordinates and structure factors (code 1YT2 (unliganded intact N-domain), 1YT1 (unliganded N-domain lacking the charged linker), 1YT0 (the ADP-soaked N-domain), and 1YSZ (NECA-soaked N-domains)) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 Y. Argon, personal communication.

2 The abbreviations used are: NECA, N-ethylcarboxamido adenosine; ER, endoplasmic reticulum.

Mediated by GRP94 and Hsp90 and their potential as therapeutic targets, the mechanism of action of these chaperones has been under intensive investigation.

Hsp90 chaperones are organized into three distinct structural domains: N-terminal, middle, and C-terminal. The N-terminal domain is the site of regulatory ligand binding, the middle domain is the likely site of client protein interaction, and the C-terminal domain contains an obligatory dimer interface. Like cytoplasmic Hsp90 (10, 11), GRP94 is regulated by nucleotide binding to its N-terminal domain (2). Hsp90 inhibitors such as geldanamycin and radiocald bind to the N-domain and compete for the ATP binding site (12, 13). Although Hsp90 chaperone activity involves cycles of nucleotide binding and hydrolysis, the mechanistic consequences of this reaction have yet to be determined. By analogy to MutL (14) and DNA gyrase (15), two members of the GHKL family to which Hsp90s belong (16), models of Hsp90 function posit that the so-called "lid" region of the N-domain (a subset of the helix 1-4-5 subdomain comprised of helices 4 and 5 and the loop that connects them; see Fig. 1) closes over the ligand binding cavity in response to nucleotide binding and/or hydrolysis. This closing of the lid is then thought to result in the dimerization of the two N-domains thereby forming a "clamp" structure whose pivot point is the obligatory dimer interface in the C-terminal domain (17). This model has been controversial, however, as the helix 4 and 5 motions have not been observed experimentally for Hsp90, and a recent kinetic study is consistent with a model where N-domain dimerization and, hence, the chaperone clamp structure, does not occur (18).

In contrast to Hsp90, recent structural studies of GRP94 have shown that adenosine nucleotide binding leads to a large conformational rearrangement in the helix 1-4-5 subdomain of the N-domain (19) compared with its counterpart in the inhibited form of the protein (see Fig. 3, A and B). This rearrangement, which results in a switch between the "closed" (inhibitor-bound) and "open" (ATP- and ADP-bound) conformations, exposes a predominantly hydrophobic surface contributed by residues Phe-78, Val-82, Met-85, Met-86, Ile-89, Ser-92, Leu-93, Tyr-94, Met-178, Gln-182, Thr-188, and Leu-191 that was shown in the structure to serve as an N-domain dimerization interface (19). The nucleotide-dependent open conformation of GRP94 was shown to arise as a consequence of a 5-amino acid insertion that extends helix 4 and that, within the Hsp90 family, is unique to GRP94 (Fig. 1). This insertion in GRP94 forms an additional pocket adjacent to the adenine binding cavity that allows binding to specific ligands such as N-ethylcarboxamido adenosine (NECA)2 (20). The insertion also correlates with a lack of ATP hydrolysis activity in the ER chaperone (21, 22). Together these suggest that GRP94 and Hsp90, al-
though sharing extensive structural and sequence homology, employ different regulatory mechanisms.

The two ligand-dependent conformations of GRP94 were detected by comparing the inhibitor-bound forms of GRP94 (radicicol, NECA, 2-Cl-dideoxyadenosine) (20) with the nucleotide-bound forms (19). However, it is likely that the physiologically relevant transition in GRP94 is between the nucleotide-bound state and the unliganded state. Similarly, to understand the mechanism of action of Hsp90 inhibitors such as geldanamycin and radicicol and to fully exploit the structure of the protein in the design of novel high affinity inhibitors, it is necessary to have a description of both the nucleotide-bound and unliganded states of the molecule. To date the structure of unliganded GRP94 has not been described.

Modeling studies that placed adenosine nucleotides into the inhibitor-bound conformation of GRP94 led to the prediction that the carbonyl oxygen of Gly-196 would have to be repositioned for nucleotide binding to occur (20). Although this has been borne out experimentally, the conformational changes in GRP94 seen by comparing the inhibitor-bound and nucleotide-bound forms are much larger than is necessary to accommodate the nucleotide (19). Moreover, in the model proposed for Hsp90, the lid elements of the N-domain (helices 4 and 5) were proposed to close over the nucleotide binding cavity in response to nucleotide binding (17). However, the experimental observation for GRP94 was just the opposite, namely that helices 4 and 5 opened away from the ligand binding cavity in response to nucleotide binding (see Fig. 3, A and B). To more closely ad-
dress the physiologically relevant transition in GRP94, we have now determined the structure of the unliganded form of the GRP94 N-terminal domain. From this structure, we show that the conformation adopted in the absence of ligand is identical to the closed conformation seen in the inhibitor-bound form. Additionally, we have soaked adenosine nucleotides into crystals of unliganded GRP94, thereby divorcing the packing of the protein into a crystalline lattice from conformational changes caused by nucleotide binding. The structure of this nucleotide-soaked crystalline complex reveals an intermediate state trapped between the open and closed conformations. Together these studies show that the nucleotide-bound and unliganded inhibitors act to stabilize the closed, unliganded conformation, and that the 5’ substituent of adenosine nucleotides is responsible for driving the conformation into the open state.

**Experimental Procedures**

**Protein Purification and Crystallization—**GRP94-(69–337)-N-propylcarboxamido adenosine complex (PDB code 1U04) was solved by molecular replacement and refined against 2.2-Å resolution data sets for ADP-soaked complex, which was processed with XDS (24). Initial phases for each GRP94-(69–337)-ADP complex were determined by molecular replacement (MolRep) using the entire protein component of GRP94-(69–337)-ADP (PDB code 1U02) except for the ADP-soaked complex GRP94-(69–337)-ADP data set that utilized only the core region (residues 69–166 and 200–337), as determined by difference distance matrix analysis (19, 25). The protein component of the GRP94-(69–337)-N-propylcarboxamido adenosine complex (PDB code 1U04) was used as the search model for the unliganded GRP94-(69–337) data. Initial models were refined iteratively by manual rebuilding in O (26) and refinement in CNS (27). In later rounds of refinement, ligands and solvent molecules were added, and restrained individual B-factors were used. Structure validation was performed using MAGE, KING, and Molprobit (28, 29). Molecular graphics were created using Pymol (Delano Scientific, San Carlos, CA). Data collection and refinement statistics are given in Table I.

**Coordinates—**Diffraction data and coordinates have been deposited in the PDB for the unliganded N-domain lacking the charged linker (1YT1), the ADP-soaked N-domain (1YT0), the NECA-soaked N-domain (1YSZ), and the unliganded intact N-domain (1YT2).

**RESULTS**

**Crystallographic and Structure Determination—**The structure of the unliganded N-terminal domain of GRP94-(69–337), in which 4 Gly residues replace the charged linker domain, was solved by molecular replacement and refined against 2.2-Å diffraction data. The protein component of the GRP94-(69–337)-NECA complex (PDB code 1U02) (20) was used as the search model. The structure of the intact, unliganded GRP94 N-domain was crystallized and solved by molecular replacement in a similar manner and refined against 3.25-Å diffraction data. Because crystals of unliganded GRP94-(69–337)-neca diffracted better than crystals of the unliganded intact domain, we used the charged linker deletion construct for most of the experiments and comparisons described in this report.

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**Table I**

| PDB code       | GRP94N41 unliganded | GRP94N41 ADP soak | GRP94N41 NECA soak | GRP94N unliganded |
|----------------|---------------------|-------------------|--------------------|-------------------|
| Source         | Raxi II             | APS 19-ID         | Raxi II            | Raxi II           |
| Data set       | r516eac             | Ded_252           | Ed208aa            | r516eac           |
| Space group    | P2 2 2 2            | P2 2 2 2          | P2 2 2 2           | C2 2 2 2         |
| a (Å)          | 65.096              | 65.910            | 65.586             | 90.321            |
| b (Å)          | 84.565              | 94.860            | 95.314             | 100.257           |
| c (Å)          | 94.999              | 42.910            | 42.857             | 64.013            |
| Wavelength (Å) | 1.54178             | 1.0083            | 1.54178            | 1.54178           |
| Resolution (Å) | 50-2.20             | 50-2.40           | 50-2.65            | 50-3.25           |
| Unigue(ref.)   | 27.356              | 10.364            | 8.553              | 4.742             |
| Completeness (%) | 99.8 (99.7)   | 93.9 (97.4)       | 98.3 (100)         | 99.7 (100)        |
| Average D90    | 14.2 (2.16)         | 15.8 (4.4)        | 14.5 (2.1)         | 13.0 (2.5)        |
| Redundancy     | 4.3                 | 3.7               | 2.9                | 3.5               |
| Rmerge (%)     | 9.8 (62.5)          | 6.8 (29.5)        | 7.5 (45.3)         | 10.7 (51.0)       |
| Resolution range (Å) | 50-2.20 | 50-2.40           | 50-2.65            | 50-3.25           |
| Refelections    | 25.575              | 10.364            | 7.672              | 4.478             |
| Non-solvent atoms | 3.557         | 1.542              | 1.672              | 1.732             |
| Solvent and hetero-atoms | 146   | 117               | 56                |
| Molecules in asymmetric units | 2   | 1                | 1                |
| Root mean square deviation from ideality | 0.00512 | 0.00687 | 0.00706 | 0.0111 |
| Bond lengths (Å) | 1.182         | 1.309              | 1.340              | 1.408             |
| R-factor (P > 2 σ) (%) | 19.4 (17.5) | 22.8 (22.6)       | 21.3 (19.2)        | 25.1 (23.4)       |
| Rfree (%)       | 24.0 (22.4)        | 26.4 (26.2)       | 26.4 (24.3)        | 29.4 (28.4)       |
previously shown that the charged linker was disordered in crystals of the intact domain (19, 20) and that its absence does not affect the structure. The dissociation constants for ligand binding to wild type GRP94 and GRP94(H900441) are the same for all ligands tested to date.3

Ligand-containing complexes were prepared from crystals of the unliganded protein by stabilizing the crystals in protein-free precipitant solutions and then soaking them in stabilizer solutions containing 5 mM ADP or 1 mM NECA. Many of the crystals soaked in ADP cracked under this treatment, and only those crystals that remained intact were used for further analysis. Soaked crystals were cryoprotected, flash-cooled, and subjected to data collection. Crystals of unliganded GRP94-(69–337/H900441) that were soaked in ADP-containing solutions diffracted to 2.4 Å and underwent a space group change from P212121 to P21212. The space group change reflects the gain of symmetry around a crystallographic dyad and corresponds to a switch from two molecules to one molecule per asymmetric unit. This change also led to a concomitant halving of the b axis. Because of these changes, molecular replacement was also used to solve the structure of the ADP-and NECA-soaked complexes, again using all or structurally conserved portions of the protein component of 1U2O. Structures were refined using CNS (27). Water and solvent molecules were added in later stages of refinement independently and without reference to the solvent configuration in the molecular replacement search model. For the ADP-soaked crystal, electron density for residues 167–195 was poor, and so these residues were omitted from the final model. Well defined electron density was observed for the ADP ligand. For the NECA-soaked crystal, clear electron density was observed for all but residues 167–169 and 184–186, which are in loop regions connecting helices 3 and 4 and helices 4 and 5, respectively. Portions of the 2Fo − Fc electron density maps for all 3 structures are shown in Fig. 2.

**The Apoprotein N-domain Adopts the Closed Conformation**—To date we have observed two distinct conformations of the GRP94 N-domain, each associated with different classes of bound ligands. The closed conformation was observed in GRP94 bound to the high affinity inhibitors NECA, 2Cl-dideoxyadenosine, and radicicol (20) as well as geldanamycin.3 The open conformation was seen in GRP94 bound to the adenosine nucleotides ATP, ADP, and AMP (19). The open and closed conformations differ in

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3 R. M. Immormino and D. T. Gewirth, unpublished data.
the relative positions of helices 4 and 5 along with helix 1 and strand 1. These rearrangements arise from the need to avert potential clashes between the carbonyl oxygen of Gly-196 in the closed conformation with the \( P^i_9251 \) and \( P^i_9252 \) phosphates of the bound adenosine nucleotide. Because Gly-196 is reoriented to avoid these clashes, helices 4 and 5 are forced to move as well, leading to the rearrangement of helix 1 and strand 1.

By analogy with Hsp90 and other members of the GHKL family, adenosine nucleotides are likely physiological ligands for GRP94. Because the binding constant for adenosine nucleotides to GRP94 is weak and ATP hydrolysis above background has not been detected (21, 22), the relevant physiological transition for the chaperone is expected to be between the nucleotide-bound form and the unliganded form. We therefore compared the structure of the unliganded form of GRP94-(69–337\( \Delta \)41) with that of the open and closed conformations. We therefore compared the structure of the unliganded form of GRP94-(69–337\( \Delta \)41) with that of the open and closed conformations. The NECA-bound GRP94-(69–337\( \Delta \)41) complex (PDB code 1U2O) was used as a representative closed complex, although similar results hold when the radicicol or geldanamycin-GRP94 complexes are used as well. We used distance difference matrix analysis (25) to align the structurally invariant regions of the unliganded protein with the open and closed conformations. As seen from the overlay of the open, closed, and unliganded structures in Fig. 3, unliganded GRP94 adopts a conformation that recapitulates the closed, or inhibitor-bound form (Figs. 3, A and C). The root mean square deviation between all \( C^\alpha \) atoms in the NECA-bound closed form and the unliganded form is 0.26 Å. Although solved at lower resolution, the structure of the intact, unliganded N-domain also adopts the closed conformation, indicating that the presence or absence of the charged linker does not affect the response to ligand binding. This suggests that in GRP94 the effect of high affinity inhibitors such as NECA, geldanamycin, and radicicol is to fix the N-terminal domain in the unliganded, closed conformation and to disfavor the open conformation. Because only the open conformation exposes the N-domain dimer interface (19), the effect of “locking” the protein in the closed conformation is to prevent GRP94 quaternary interactions.

**Fig. 3. Comparison of GRP94-ligand complexes with unliganded GRP94.** A, overlay of GRP94-(69–337\( \Delta \)41)-NECA (PDB code 1U2O) with unliganded GRP94-(69–337\( \Delta \)41). The NECA complex is colored blue, and the unliganded (apo)protein is colored green. B, overlay of GRP94-(69–337\( \Delta \)41)-ADP (PDB code 1TC6) with unliganded GRP94-(69–337\( \Delta \)41). The ADP complex is colored gold and is representative of an N-domain in the open conformation. C, stereo \( C^\alpha \) trace of GRP94-(69–337\( \Delta \)41)-NECA, GRP94-(69–337\( \Delta \)41)-ADP, and unliganded GRP94-(69–337\( \Delta \)41) N-domains. Colors are as in A and B above. Structurally invariant regions were used to align the three molecules.
Soaked-in Nucleotides Lead to an Intermediate Conformation of the N-domain—Although not yet observed experimentally, models of Hsp90 action posit that the helix 4/5 lid element closes over the nucleotide binding cavity in response to nucleotide occupancy (17). Two surprising observations, however, arose from the co-crystal structures of the ATP-, ADP-, and AMP-bound forms of GRP94 that distinguish GRP94 from Hsp90. First, a large conformational shift was seen in helices 1, 4, and 5 in response to nucleotide binding (19). Second, these elements opened away from, rather than closed over, the binding cavity in response to the bound nucleotide. With regard to the first observation, modeling showed that potential clashes between the protein and the phosphates of the nucleotide could be relieved by moving the carbonyl oxygen of Gly-196 by as little as 1.7 Å away from the nucleotide. Instead of this modest rearrangement, however, the co-crystal structure of nucleotide-bound GRP94 revealed a 10.8-Å displacement of the carbonyl oxygen of Gly-196. This extraordinary motion of Gly-196 resulted in the large scale rearrangement of helices 1, 4, and 5, the incorporation of strand 1 into helix 1, and the exposure of an N-domain dimer interface. Because both the magnitude and the direction of these motions were unexpected and did not correspond to the Hsp90 model, we sought to see whether a change in the protein environment would yield a different outcome when nucleotides bound. In addition, we asked whether the motions we observed were the direct effect of nucleotide binding or whether nucleotides bound only to proteins that had first adopted the open conformation. To answer these questions we soaked ADP into stabilized crystals of unliganded GRP94-(69–337/H9004). We used crystals of this construct because they diffracted better than crystals of the intact N-domain. Because the protein was already incorporated into a crystal lattice before the introduction of nucleotide, any rearrangements of the protein in the soaked crystal would be due to nucleotide binding rather than selective trapping of conformational states that were compatible with a crystal lattice.

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The structure of the ADP-soaked GRP94 is shown in Fig. 4A. Nucleotide binding displaces the polyethylene glycol precipitant and the labile solvent molecules from the adenine binding cavity, and clear electron density was observed for the bound ADP (Fig.

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**FIG. 4. Structure of ADP-soaked GRP94 N-domain crystals.** A, overall structure shown in a ribbon drawing. Disordered regions are indicated by a dashed line. B, comparison of the positions of Gly-196. The structures of unliganded (green), ADP-co-crystallized (gold), and ADP-soaked (blue) N-domain are shown. Distances between the α carbons of Gly-196 in the three crystal structures are shown. The ADP position relative to the binding cavity and the bulk of the protein is the same in both the soaked and co-crystallized forms. C, packing analysis of GRP94-ADP complexes. GRP94-ADP complexes in the open conformation, as seen in the structure of the co-crystal complex, were modeled into crystals of the ADP-soaked complex. Severe clashes are seen between helix 1 of symmetry-related molecules, indicating that this structure is not compatible with this crystal lattice. D, packing analysis of ADP-soaked N-domain. A space-filling surface is shown over the ribbon drawing. In this crystal form helix 1 from symmetry-related molecules are packed against one another and cannot move without disrupting the crystal lattice.
The soaked-in ADP ligand adopts the same compact conformation seen in the co-crystal structure of GRP94-ADP, and the soaked-in ligand makes the same direct and solvent-mediated hydrogen bonds as in the co-crystallized complex.

As in the co-crystallized complex, ligand-induced rotations around Gly-198 lead to the displacement of the carbonyl oxygen of Gly-196 from its position in the unliganded or inhibitor-bound complexes. However, in the ADP-soaked complex, the rotation around Gly-198 is incomplete ($\Delta \phi$ of $+12\text{ to }17\text{ degrees compared with }+144\text{ to }160\text{ degrees}$ for the full rotation seen in the co-crystallized complex), with the result that the carbonyl oxygen is displaced from its position in the closed conformation by only 5.7 Å compared with 10.8 Å in the co-crystal structure (Fig. 4B). The ADP-soaked crystal structure, therefore, represents an intermediate state between the open and closed conformations. The same intermediate state was observed in two completely independent determinations of the structure of ADP-soaked crystals.

In the co-crystal structure of nucleotide-bound GRP94, helices 4 and 5 are rotated outward compared with their counterparts in the closed conformation. In the ADP-soaked crystal form, helices 4 and 5 are disordered, an observation that is seen in both independent structure determinations of ADP-soaked crystals. Importantly, however, despite these limitations the motions we observe in response to soaked-in ligand are consistent with a rotational opening of the helix 4/5 lid elements, since any other types of motions, for example ones that result in the closing of helices 4 and 5, would place Gly-196 on a different trajectory. Earlier proposals for helix 4/5 motions in Hsp90, based on a mechanistic analogy to the motions of similar elements in MutL and DNA gyrase, suggested that the helix 4/5 lid elements in Hsp90 would close in response to nucleotide (17). The fact that we have observed an intermediate state that is also consistent with the opening motions seen in the ATP, ADP, and AMP co-crystal structures (19) indicates that the GRP94 helix 4/5 lid does not close in response to nucleotide binding.

Despite the partial opening movement of Gly-196 in the ADP-soaked crystal form, helix 1 and strand 1 are maintained in the closed conformation. A comparison of the lattice contacts in the unliganded-, ADP-soaked-, and ADP-co-crystal structures explains why the fully open conformation is not observed. As seen in Fig. 4C, modeling GRP94 in the open conformation into the lattice of the unliganded protein reveals that helix 1 and strand 1 would form unacceptable overlaps with symmetry mates in the crystal lattice of the closed form. In the ADP-soaked intermediate form, helix 1 is constrained from moving by lattice contacts (Fig. 4D). The 5.7-Å displacement of the carbonyl oxygen of Gly-196, therefore, is likely to be the maximum movement allowable before helix 1 and strand 1 must be rearranged. This lattice constraint to further motions can also explain why a substantial fraction of the ADP-soaked crystals fractured upon being soaked with ADP. Presumably the transition to the fully open conformation went to completion in these crystals, resulting in the disruption of the crystal lattice.

The fact that Gly-196 movement in response to ADP binding is independent of helix 1 and strand 1 movements shows that nucleotide binding and the need to displace Gly-196 are the precipitating events that lead to the rearrangement into the open conformation.

To show that the conformational changes seen in the ADP-soaked crystal were due specifically to the nucleotide, we also soaked the inhibitor NECA into the unliganded GRP94 crystals and solved this structure. We used NECA for these control experiments because NECA is also based on an adenosine scaffold and differs from ADP only in its 5’ substituent, the moiety that drives conformational rearrangements. As seen from the overlaid structures in Fig. 5, the conformation of the GRP94 N-domain in complex with NECA that has been soaked into the crystalline protein is essentially identical to that of the co-crystallized GRP94-NECA complex and to that of the unliganded protein. We conclude, therefore, that the conformational changes seen in the ADP-soaked crystal are due to nucleotide binding specifically and not from rearrangements arising from general ligand binding. Helices 4 and 5 are also mostly ordered in the NECA-soaked complex, indicating that introduction of ligand into the pre-formed crystalline lattice does not lead to crystallographic disorder of these elements.

**Conserved Solvent Arrangements in the Adenine Binding Cavity—**In every GRP94-ligand complex structure determined to date, all but one of the protein-ligand interactions within the adenine binding cavity are mediated by intervening water molecules. In the unliganded form of the protein, the adenine binding cavity is occupied by an ordered network of water and polyethylene glycol molecules that arise from the crystallization medium (Fig. 6). Despite the absence of any high affinity ligand, three of these water molecules, shown in purple in Fig. 6, are conserved between the structures of the unliganded protein and the liganded complexes. The first of these water molecules forms a hydrogen bond with the carbonyl oxygen of Leu-104, the second bridges the carboxylate side chain of Asp-149, the γ-hydroxyl of Thr-245, and the amino nitrogen of Gly-153, and the third is coordinated by the carboxylate group of Asp-149 and bridges the interaction between this carboxylate and the carbonyl oxygen of Leu-104 via the first conserved water. Because they are conserved among all liganded and unliganded states of the protein, these water molecules must be considered part of the intrinsic structure of the ligand binding cavity. Any structure-based design of high affinity ligands for GRP94 and other Hsp90s will thus have to include these water molecules as part of the protein-ligand interface.

**Discussion**

In this report we have shown that the structure of the unliganded N-terminal domain of GRP94 adopts the closed conformation that is also observed when the N-domain is bound to GRP94 inhibitors. This conformation differs from the open, nucleotide-bound conformation in the disposition of helices 1, 4, and 5 and strand 1. The structure of the unliganded form of the regulatory domain of the chaperone completes the picture of the major conformational states adopted by the N-domain of GRP94. Together these structures support a model where ligands dictate GRP94 conformation, promote or inhibit a chaperone.
erone-clamping action via the N-terminal dimerization site, and thereby regulate chaperone action. As seen in Fig. 7, the nucleotide-bound state adopts a conformation that leads to N-domain dimerization and the formation of the chaperone clamp. In the absence of the adenosine nucleotide, the N-domain shifts from the open to the closed conformation. This conformation is incompatible with N-domain dimerization, leads to the opening of the chaperone clamp, and likely represents an inactive state of the molecule. High affinity inhibitory ligands such as geldanamycin and radicicol stabilize the closed state of the N-domain and, hence, the opening of the chaperone clamp, which is now shown to be equivalent to the ligand-free state. The effect of inhibitors thus appears to be the stabilization of a GRP94 conformation that is incompatible with N-terminal dimerization. Although the mechanism of chaperone interaction with client proteins has yet to be elucidated, studies of DNA gyrase (15) and MutL (14), other members of the GHKL family, implicate a second dimer interface in addition to the high affinity dimer interface in the C-terminal domain for activity.

Structural studies have shown that the lid elements of DNA gyrase and MutL respond to nucleotide binding by closing over the ligand binding region and stabilizing the bound nucleotide. Experimental evidence for similar helix 4/5 lid motions in the Hsp90 system has been mixed, however. On the one hand, mutants of yeast Hsp90 that increase the rate of ATP hydrolysis map to regions of the N-domain that interact with the helices 4 and 5 (17). On the other hand, unlike the GyrB or MutL systems, direct visualization of alternate helix 4/5 conformations in the Hsp90 system has not been forthcoming. Further confusing the issue was the observation in GRP94 that helices 4 and 5 do move in response to nucleotide binding, but instead of closing over the bound nucleotide as in MutL and DNA gyrase, these helices opened away from the ligand and did not function in the classic sense as a lid at all (19). Because of the uncertainty surrounding this point, we sought to confirm both the effect of nucleotide binding and the direction of the helix 4/5 motions. Indeed, as reported here, soaks of adenosine nucleotides into the crystals of the unliganded protein reveal a conformation of the GRP94 N-domain that represents a structural intermediate between the fully closed and fully open states of the domain. By separating the incorporation of the protein into a crystalline lattice from the conformational change driven by nucleotide binding, these structures show that helices 4 and 5 do in fact undergo an opening motion in response to ligand binding. Moreover, because soaking NECA into the crystals of the unliganded protein did not result in a conformational change, these experiments also show that it is the identity of the bound ligand rather than ligand binding per se that drives the conformational changes in the GRP94 N-domain. These experiments demonstrate that it is possible to observe alternate conformations of the helix 4/5 lid elements in a member of the Hsp90 chaperone family and also suggests that the failure to observe these in the N-domain of cytoplasmic Hsp90 may mean that such motions either do not occur, require elements from the intact chaperone, or depend on the partici-
pation of co-chaperone proteins. Support for the idea that elements outside the N-domain may influence N-domain structure comes from a recent crystal structure of the N and middle domains of E. coli HtpG, which showed that a bound nucleotide interacts with both domains (30). The fact that distinct, unique nucleotide-driven conformational changes are observed in the isolated N-domain of GRP94, however, clearly distinguishes the GRP94 system from that of Hsp90.

GRP94 and cytoplasmic Hsp90 are paralogs that arose via gene duplication early in the evolution of the eukaryotic cell (31). The gene duplication event that gave rise to GRP94 allowed for chaperone specialization through the evolutionarily conserved insertion of 5 amino acids in the N-domain of GRP94 in the loop that connects helices 4 and 5. The effects of this insert are revealed structurally in the different conformational responses of GRP94 and Hsp90 to adenosine nucleotide binding. In particular, structural studies of the Hsp90 N-terminal domain have detected no significant conformational changes in response to ligand compared with GRP94, which undergoes dramatic ligand-dependent structural rearrangements. Indeed, conformational variation in the N-terminal regulatory domain appears to be the structural hallmark of GRP94 that distinguishes the ER chaperone from its cytoplasmic cousin. In addition to the nucleotide-dependent conformational shift, we have also observed significant non-ligand-dependent conformational changes in the GRP94 N-domain. As seen in Fig. 8, two different conformations are observed in the interaction of strand 1 with strand 6 in different crystal forms of the unliganded GRP94 N-domain, and the same comparison reveals a 7° shift in the orientation of the helices 4 and 5. Because both of these elements are implicated in the transition from the closed to the open conformation of the GRP94 N-domain, these structural variations are consistent with a model where conformational changes originating from within the N-domain are the predominant mode of GRP94 signaling and regulation. Importantly, conformational variations in strand 1 or helix 1, which are mechanistically coupled to changes in lid orientation and dimerization and which in vitro can affect the rate of ATP hydrolysis (32), have not been seen in any of the 27 structures of yeast or human Hsp90 N-domains determined to date.

Together, these differences are likely to reflect the paralog-specific role of adenosine nucleotides in chaperone activity. In Hsp90, chaperone activity is closely coupled to ATP hydrolysis and, significantly, with co-chaperone binding. In GRP94, on the other hand, ATP hydrolysis above background levels has not been detected (21, 22), and co-chaperones have not been identified for the ER paralog. Instead, GRP94 exhibits large scale ligand-dependent conformational changes. It is thus tempting to speculate that the conformational changes that accompany ligand binding to GRP94 may functionally substitute for the co-chaperone interactions seen in cytoplasmic Hsp90.

Given that Hsp90 and GRP94 exhibit significant sequence homology and likely have similar roles in their respective cellular compartments, it may seem puzzling that the two paralogs evolved different mechanisms of action. However, chaperoned protein folding differs between the ER and the cytoplasm in several key respects. Far fewer client proteins for GRP94 have been identified than for Hsp90, and this restricted set of clients may allow the ER to make do with a simpler system than that which evolved in the cytoplasm. Moreover, Hsp90 and GRP94 differ in the temporal nature of their client protein interactions. Complexes between Hsp90 and client proteins such as the steroid receptors can be very long-lived (33), whereas client proteins reside only briefly in the ER lumen before exiting into the secretory pathway. The streamlined ER apparatus may, thus, reflect the more transient nature of the chaperone-client interactions in this organelle. Finally, Hsp90 and GRP94 are among the most abundant proteins in the cell,
an extravagance that is energetically costly. In light of the different folding requirements of the two cellular compartments, it is not surprising that Hsp90 and GRP94 have diverged to extract efficiencies from so costly an apparatus.

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