Structure of the N-terminal Domain of GRP94

BASIS FOR LIGAND SPECIFICITY AND REGULATION*

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GRP94, the endoplasmic reticulum (ER) parolog of the chaperone Hsp90, plays an essential role in the structural maturation or secretion of a subset of proteins destined for transport to the cell surface, such as the Toll-like receptors 2 and 4, and IgG, respectively. GRP94 differs from cytoplasmic Hsp90 by exhibiting very weak ATP binding and hydrolysis activity. GRP94 also binds selectively to a series of substituted adenosine analogs. The high resolution crystal structures at 1.75–2.1 Å of the N-terminal and adjacent charged domains of GRP94 in complex with N-ethylcarboxamidoadenosine, radicicol, and 2-chlorodideoxyadenosine reveals a structural mechanism for ligand discrimination among hsp90 family members. The structures also identify a putative subdomain that may act as a ligand-responsive switch. The residues of the charged region fold into a disordered loop whose termini are ordered and continue the twisted beta sheet that forms the structural core of the N-domain. This continuation of the beta sheet past the charged domain suggests a structural basis for the association of the N-terminal and middle domains of the full-length chaperone.

The hsp90 family of molecular chaperones are ligand-regulated proteins that participate in the conformational maturation of protein substrates involved in diverse cellular activities ranging from cell signaling to bacterial recognition (1, 2). They are also overexpressed in response to cell stress events, including heat shock, starvation, and oxidation. Cytoplasmic Hsp90α and -β play central roles in cell signaling by guiding the maturation of steroid hormone receptors and proto-oncogenic kinases. Ansamycin antibiotic inhibitors that target the hsp90 family members, such as geldanamycin, radicicol, and herbimycin B, disrupt this maturation process and consequently display potent anti-cancer activity (3–9). The endoplasmic reticulum paralog of cytoplasmic Hsp90, called GRP94 (also known as gp96), shepherds the folding of membrane-bound proteins, including the Toll-like receptors and subclasses of integrins (10), as well as secreted proteins such as IgG (11, 12). Cells deficient in GRP94 are unresponsive to microbial stimuli (10). GRP94 has also been identified as a tumor rejection antigen (13) and has recently been shown to elicit suppression of tumor growth and metastasis by a mechanism independent of bound peptides (14). Given their central importance in the biology of protein folding and the cellular stress response, and their potential as targets in a variety of therapeutic strategies, the mechanism by which hsp90 chaperone activity is regulated, and the chemistry of their interactions with client substrates is under intensive investigation.

GRP94 and Hsp90 exist as obligate homodimers, with each subunit consisting of an N-terminal domain, a charged region, and a C-terminal dimerization domain. Because of its relatively weak affinity (∼100 μM), the binding of ATP and ADP to Hsp90 was conclusively established only with the determination of the co-crystal structures of the N-domain in complex with bound nucleotide (15). Other crystal structures of the N-domain of Hsp90 in complex with the ansamycin antibiotics geldanamycin (16) and radicicol, and other inhibitors (17), showed that despite their lack of structural similarity to ATP these ligands bind to the same domain and function as adenosine mimetics. The N- and C-domains are connected by a 40- to 60-residue charged region that varies in length and sequence among species and family members. Rotary shadowing electron microscopy studies have suggested that the charged region acts as a flexible tether that links the compactly folded N- and C-domains (18–23).

By analogy with DNA gyrase (24) and MutL (25), other members of the GHKL family (26) to which Hsp90 belongs, it has been proposed that hsp90 chaperone activity is linked to an N-domain conformational change that is coupled to ATP binding and hydrolysis (27, 28). Indeed, a slow ATP hydrolysis activity has been detected in Hsp90 (29–31). Mechanistic explanations of how ATP binding and hydrolysis are coupled to Hsp90 activity have been hindered, however, by the lack of any observed change in the conformation of the N-domain in response to ligands. The crystal structures of the Hsp90 N-domain, either in the apo form or in complex with ATP, ADP, and inhibitors such as geldanamycin, radicicol, and others are essentially superimposable (15–17, 30, 32). The complete protein, rather than just the isolated N-domain, appears to be required for ATP hydrolysis (30, 33), suggesting that conformational rearrangements resulting in a close interaction with the C-domain are important for Hsp90 activity. However, the manner in which N- and C-domains associate across the flexible linker and the interfaces participating in oligomerization remain unknown. Interactions with co-chaperones may also be important catalysts for ATP hydrolysis in Hsp90s from some species as well (34).

Despite having over 50% sequence identity in their N-domains and complete conservation of their ligand binding cavities, Hsp90 and GRP94 differ in their interactions with regulatory ligands and may thus differ mechanistically as well.

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Although GRP94 responds to ansamycin inhibitors in a manner analogous to Hsp90, indicating ligand regulation (35–37), ATP hydrolysis above background levels has not been demonstrated for GRP94 (38), nor have co-chaperones or partner proteins been identified for this paralog. Domains of Hsp90 and GRP94 also do not functionally substitute for one another (10). In addition, although Hsp90 binds ADAP with micromolar affinity (15), the binding constant for these ligands to GRP94 is estimated to be several millimolar (38). The difference in ligand regulation between GRP94 and Hsp90 is further highlighted by their interactions with 5′-N-ethylcarboxamidoadenosine (NECA),1 a broad-spectrum adenosine A3 receptor antagonist. Biochemical screens for NECA-binding activity identified GRP94 as the prominent cellular target of this ligand, with an equilibrium dissociation constant of 200 nM (39, 40). NECA has no detectable binding affinity for cytoplasmic Hsp90α, however (38).

The functional differences between GRP94 and Hsp90 underscore the fact that many aspects of hsp90 biology remain to be addressed, including the mechanism of selective regulation of the cellular paralogs, the role of ATP in GRP94 where ATP hydrolysis has proven difficult to detect, and the structural basis for tertiary interactions between hsp90 domains and quaternary associations between intact chaperones. To understand the mechanism for selective ligand responsiveness in GRP94, we have solved the structure of the N-domain of GRP94 in complex with the GRP94-specific ligand NECA, as well as with the high affinity but non-selective ligands radicicol and 2-chloro-ribbonoxadiazonoside (2ClddA). We show that an intrinsic conformational difference between cytoplasmic Hsp90 and GRP94 accounts for selectivity in ligand binding. We also show that the 5′-substituent of NECA occupies a previously unidentified second pocket adjacent to the adenine-binding cavity. This structural difference arises in large part from a 5-amino acid insertion unique to GRP94 whose structural role until now has been unknown. These structures also identify a subdomain within the N-domain that has the potential to act as a ligand-responsive conformational switch in GRP94. Finally, to understand the structural relationship between the N- and C-domains of GRP94, the constructs we used for crystallization have included the charged region. We showed here that this region forms a disordered loop whose end orients surprisingly, to form an ordered, stable interaction with the body of the N-domain. This provides evidence for an intimate interaction between the N- and C-domains of the protein.

**EXPERIMENTAL PROCEDURES**

**Protein Purification—**Canine GRP94 (residues 69–337) was overexpressed in *Escherichia coli* as a GST fusion. BL21(DE3)pLysS cells harboring the expression plasmid were grown to an \

\[\text{A}_{600}\text{nm} \text{ at } 37\,\text{°C} \text{ and induced by the addition of isopropyl-1-thio-\beta-D-galactopyranoside to a final concentration of 0.1 mM. The cells were harvested after 3 more h of shaking, and the cell pellet was lysed in 25 mM Tris, pH 8.0, 350 mM NaCl, 5 mM DTT, 1 mM phenylmethylsulfonyl fluoride by three passes through a Microfluidics M110L homogenizer. The cell lysate was cleared by centrifugation at 25,000 \times g, and the supernatant was applied to a column of glutathione-agarose (Sigma) in lysis buffer. GST-GRP94-(69–337) was eluted with a buffer containing 10 mM glutathione, 50 mM Tris, pH 8.0, 200 mM NaCl, 1 mM DTT. The fusion protein was diluted to 2.5 mg/ml and cleaved by the addition of thrombin (Hematologic Technologies) at an enzyme:protein ratio of 1:1000 (w/w) for 3.0 h at room temperature while being dialyzed against 50 mM Tris, pH 7.6, 50 mM NaCl, 1 mM DTT. The cleaved mixture was applied to a Q-Sepharose Fast Flow column (Amersham Biosciences) and eluted with a 50–800 mM NaCl gradient in 50 mM Tris, pH 7.6, 1 mM DTT. The fractions containing intact GRP94-(69–337) were combined and concentrated in a Centriprep-10 (Millipore) and further purified by gel filtration (Superdex 75, Amersham Biosciences) and high performance ion exchange (Resource Q, Amersham Biosciences) chromatography, both in 50 mM Tris, pH 7.6, buffers. Purified GRP94-(69–337) was concentrated by ultrafiltration in a Millipore Ultrafree 4–30 mg/ml buffer comprised of 50 mM Tris, pH 7.6, 100 mM NaCl, supplemented in small aliquots at –80 °C. GRP94-(69–337)/ADAM0) was purified in a similar manner, except that the final Resource Q step was omitted.

**Ligands and Protein-Ligand Complexes—**NECA and radicicol were purchased from Sigma. 2-Chloro-ribbonoxadiazonoside (2ClddA) was obtained from the Open Chemical Repository of the Drug Therapeutics Program at the NCI, National Institutes of Health (Bethesda, MD). Grainsomes were prepared by 3- to 5-fold molar excess of concentrated ligand (10–50 μM) in MEGSO to the 30 mg/ml protein solution and used without further purification.

**Crystallography and Data Collection—**GRP94-(69–337) ligand complexes were crystallized at 18 °C by the hanging drop vapor diffusion method. Two μl of protein-ligand complex was mixed with an equal volume of reservoir solution, which consisted of 10 mM Tris, pH 7.6, 200–300 mM MgCl2, and 35–45% PEG 400 for the NECA, 2ClddA, and Δ40 complexes. The reservoir for the radicicol complex was the same except that PEG 550 monomethylether was substituted for PEG 400. Diffraction quality crystals with typical dimensions of 0.6 × 0.2 × 0.1 mm grew in less than 1 week.

**Crystals of the NECA complex were strengthened by vapor diffusion crosslinking with glutathione following the procedure of Lusty (41) using 5 μl of a 25% glutaraldehyde solution in a microbridge for 30 min.**

Following cross-linking the drop containing the crystals was placed over a fresh reservoir solution containing 100 mM Tris, pH 7.6, 220 mM MgCl2, 43% PEG 400 and gradually stabilized with this solution. Stabilized crystals were removed from the drop in nylon loops and flash frozen in liquid nitrogen. Crystals of the radicicol and Δ40 complexes were removed directly from the mother liquor and flash frozen in a stream of N2 gas cooled to –170 °C. Crystals of the 2ClddA complex were equilibrated into reservoir solution that had been supplemented with glycerol to a final concentration of 20%, removed from the drop in loops and flash frozen in liquid nitrogen.

Diffraction data sets used to obtain initial phases for the NECA complex were collected at 113 K on a home source using CuKα x-rays. High resolution data were collected at 100 K on beamlines 19BM or 14-IDB at the Advanced Photon Source using charge-coupled device detectors. All data was indexed and reduced using HKL2000 (42).

**Structure Determination and Refinement—**The structures of the GRP94-(69–337)/ligand complexes were solved by molecular replacement with the CNS (43). Initial phases for the NECA complex were obtained by molecular replacement using a 2.75-Å home source data set processed in space group C2 (Native 1, 97.6% complete, I/σo = 2.2 last shell, Rmerge = 0.057), with the search model consisting of the backbone atoms only of the N-domain of yeast Hsp90 (PDB code 1AMW). The two GRP94 molecules in the asymmetric unit were located sequentially in the translation search. Electron density maps calculated from this solution revealed good density for the model through Hsp90 residue 111 (residue 181 in the GRP94 numbering). Subsequent rounds of simulated annealing refinement and manual rebuilding with O (44) against a 1.9-Å Advanced Photon Source 14BM synchrotron data set (Native 2, 89.7% complete, I/σo = 2.1 last shell, Rmerge = 0.109) using NCS to build the asymmetric unit followed the course of the GRP94 polypeptide from residues 73–286 and 331–337, as well as the NECA ligand. Final refinement was carried out against a 1.75-Å C2222 data set (Native 3) using a maximum likelihood target, bulk solvent correction, and individual restrained B factor correction. The radicicol and 2ClddA complexes were solved by molecular replacement using the polypeptide from the partially refined NECA crystals for the search model and automatically solved using the program Molrep applied to the NECA complex. The GRP94-(69–337)/40NECA complex, which has two molecules in the asymmetric unit, was solved by molecular replacement using a partially refined P2_1_2_1 complex as the search model, which also has two molecules in the asymmetric unit, and was refined as described above. Refinement statistics are given in Table 1.

**Table 1.**
**RESULTS**

Crystallography and Structure Determination—The structures of three complexes between GRP94-(69–337) and NECA, radicicol, and 2ClddA were solved and refined at 1.75-, 1.85-, and 2.1-Å resolution, respectively. The GRP94-(69–337) protein domain was crystallized in the presence of a 5-fold molar excess of ligand. The structure of the GRP94-NECA complex was solved by molecular replacement using the backbone atoms of the GRP94-(69–337)–radicicol and GRP94-(69–337)–2ClddA complexes were solved by molecular replacement using the backbone atoms of the GRP94-(69–337)–NECA complex as the search model. In all three structures the charged domain residues 287–337 (Met154, Asn162, Gly196, Phe199, and Thr245) are completely conserved and have an equivalent location relative to the rest of the N-domain. Helices 1, 4, and 5 constitute a subdomain of GRP94 that has a different orientation relative to the rest of the N-domain than the equivalent region in Hsp90. Excluding this region from the comparison reduces the r.m.s. deviation between GRP94 and Hsp90 to 0.90–1.1 Å for all backbone atoms, indicating a highly similar fold. The 10 residues that make up the adenine-binding cavity (Leu104, Asn107, Ala111, Asp149, Gly153, Met154, Asn162, Gly196, Phe199, and Thr245) are completely conserved between Hsp90 and GRP94 and, with the exception of Gly196, are not in the helix 1-4-5 subdomain. The r.m.s. deviation for just these 10 residues (all atoms) is 0.89 Å with yeast Hsp90 and 1.27 Å with human Hsp90.

NECA Binding Is Stabilized by Interaction with Two Adjacent Cavities in GRP94—NECA is an adenine-based nucleotide analog that binds selectively to GRP94, and not Hsp90, with a KD of 200 nM (38, 40). Selective ligands such as NECA may aid in the elucidation of the specific role of GRP94 in the cell and serve as a scaffold for the design of high affinity modulators of GRP94 activity. To understand the basis for NECA binding to GRP94, we determined the structure of the N-domain of GRP94 in complex with NECA. A schematic drawing of the interac-

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**Table I**

| Complex          | Data set          | Source/detector | Space group | Resolution (Å) | Last shell (Å) | Unique reflections | % completeness | Average I/oI (last shell) | Redundancy | \( R_{merge} \) | \( R_{free} \) |
|------------------|-------------------|-----------------|-------------|----------------|----------------|-------------------|---------------|-------------------------|------------|----------------|----------------|
| 40/NECA          | Native            | APS 19BM/CCD    | C222         | 89.20, 99.18, 63.07 | 1.81–1.75     | 28627             | 99.7 (97.9) | 36.3 (1.89)            | 7.3        | 0.043 (0.293) | 0.055 (0.440) |
| 2ClddA           | Native            | APS 19BM/CCD    | C222         | 86.75, 99.37, 63.30 | 1.92–1.85     | 23549             | 99.0 (96.5) | 48.2 (2.9)             | 10.9       | 0.055 (0.440) | 0.054 (0.482) |
| Radicicol        | Native            | APS 14IDB/CCD   | C222         | 87.01, 98.97, 63.25 | 2.18–2.10     | 16501             | 98.2 (96.3) | 48.2 (2.9)             | 8.2        | 0.044 (0.238) | 0.049 (0.238) |
| NECA             | Native            | Ks109b5a        | P2\(_{1}\), 2\(_{1}\), 2\(_{1}\) | 65.30, 84.20, 94.09 | 2.18–2.10     | 27334             | 87.8 (50.2) | 40.4 (4.54)            | 6.3        | 0.049 (0.238) | 0.049 (0.238) |

**Fig. 1.** A, representative electron density in the GRP94-NECA complex. A sigma A-weighted 2\( F_c \) – \( F_o \) map contoured at 1.3 \( \sigma \) around the NECA is shown. The NECA atoms were omitted from the map calculation. B, chemical structure of ligands described in this report.
Two regions of GRP94 are observed to accommodate NECA binding (Fig. 2A). The first, as expected, is the adenine-binding cavity first identified in complexes between Hsp90 and adenosine nucleotides (15). The adenine moiety of NECA fits into this cavity, and its interactions with GRP94 recapitulate those seen between the adenine and the Hsp90 protein in Hsp90/H18528-ATP/ADP complexes (Fig. 2C).

The second region of GRP94 that interacts with NECA is a newly revealed pocket that is adjacent to the adenine-binding cavity (Fig. 2A). The 5’ N-ethylcarboxamido moiety of NECA lodges into this second pocket (Fig. 2B) and is stabilized by a direct hydrogen bond from the backbone carbonyl oxygen of Asn162 to N5’ and by van der Waals interactions between the ethyl carbons and the side chains of Val197 and Tyr200. Modeling experiments (not shown) based on the structure of GRP94-NECA complex show that this partially hydrophobic binding pocket could accommodate an additional atom on the end of the N5’ substituent, thereby potentially increasing the number of productive interactions between the ligand and the protein. In the crystal structure of Hsp90 this second pocket was not identified as a potential ligand binding region. This is because, when bound to Hsp90, the 5’-β-phosphate of ATP adopts a trajectory that directs it away from the potential second pocket in Hsp90 (Fig. 2, B and C). In addition, as we
discuss later, a comparison with the GRP94 structure shows that the conformation of Hsp90 restricts ligand access to the potential second pocket.

**GRP94 Interactions with Radicicol**—Radicicol, like geldanamycin, functions as an adenosine mimetic in Hsp90 and binds tightly into the adenosine binding cavity of the N-domain (49, 50). To see if these interactions were maintained in GRP94, we determined the structure of the GRP94 N-domain in complex with radicicol. As expected from the conserved residues lining the adenine binding cavity, the interactions between radicicol and GRP94 recapitulate those seen between Hsp90 and radicicol (17). Interestingly, although radicicol binding to GRP94 has been reported to require the charged region (50), no interactions are observed between radicicol and residues of this region in the structure. Neither were the interactions between radicicol and the GRP94 N-domain altered in the structure, determined at 2.2 Å, of a complex between radicicol and a GRP94 construct lacking the charged region (not shown). These observations may be reconciled in light of the GRP94 structure presented here by noting that the truncations at residues 258 and 280 in the GRP94 construct used to test radicicol binding to the charged region (50) also remove helix 6 and strand 8 of the N-domain core. The removal of these secondary structural elements is likely to destabilize the fold of the protein and disrupt all ligand binding.

The **GRP94 Conformation Is Not Induced by NECA**—Because the 5′ substituent of NECA is different from that of ATP and ADP, we wondered if the observed GRP94 conformation and second binding pocket are intrinsic to GRP94 or are induced by the need to accommodate the 5′ substituent of NECA. To test this idea, we compared the structures of GRP94 in the NECA-bound form with that of the radicicol- and 2ClddA-bound forms. Both radicicol and 2ClddA utilize the adenine-binding cavity of GRP94 but lack the 5′ substituent (Fig. 1B) that would interact with the second binding pocket. Thus, by this reasoning, if the GRP94 conformation is induced by the 5′ substituent of NECA, complexes of GRP94 with ligands lacking such 5′ substituents, like radicicol and 2ClddA, should reveal a different conformation in GRP94. To detect such rearrangements, either local or global, we used difference distance matrix analysis (48) to compare the relative α-carbon positions in the three GRP94·ligand complexes. This analysis, however, shows no structural rearrangements between GRP94·NECA and GRP94·radicicol and GRP94·2ClddA (not shown), and the greatest overall r.m.s. backbone deviation between any of the pairs of structures is only 0.44 Å. From this we conclude that the conformation of GRP94 we observe in our crystal structures is not induced by the 5′ substituent of the NECA ligand.

**Conformational Differences between GRP94 and Hsp90**—Because the residues surrounding the adenine binding cavity and second pocket in GRP94 and Hsp90 are almost completely conserved, it was puzzling that NECA binds to GRP94 and not Hsp90. This suggested that there might be a conformational difference between GRP94 and Hsp90. Using difference distance matrices (48), we found that there was one significant cluster of divergence greater than 2 Å between GRP94 and Hsp90. This subdomain consists of helices 1, 4, and 5 of GRP94 (residues 80–95 and 164–198 corresponding to yeast Hsp90 residues 10–25 and 94–123). To visualize this, we used the non-helix 1-4-5 subdomain regions of the molecule as the basis for aligning GRP94 and Hsp90. As seen in Fig. 3A, there is an en bloc conformational shift of the helix 1-4-5 subdomain in GRP94 compared with cytosolic Hsp90. This shift alters the position of the residues in the vicinity of the second binding pocket and, as we discuss later, determines the selectivity for NECA as well as ATP. This conformational difference involves many of the same residues but does not correspond structurally to the “closed” form of apo human Hsp90 (16). Although we used the Hsp90·ADP and GRP94·NECA complexes for this particular comparison to highlight the differences in ligand binding position between ADP and NECA, the validity of this analysis derives from that fact that a comparison of the Hsp90·radicicol and GRP94·radicicol complexes (not shown) reveals the same conformational difference seen between the Hsp90·ADP and GRP94·NECA complexes. This exploits the observation that the structure of the protein in the Hsp90·radicicol complex is identical to that in the Hsp90·ADP complex, and the structure of the protein in the GRP94·radicicol complex is identical to that in the GRP94·NECA complex, thus controlling for the difference between the NECA and ADP ligands.

**Structural Origins of the Distinct GRP94 Conformation**—The N-domains of all known metazoan GRP94s contain a 3′-to-5-amino acid insertion (182QEDGQ186 in dog GRP94) that is not found in the cytoplasmic Hsp90s (Fig. 3D). This insertion occurs in the helix 1-4-5 subdomain (Fig. 3A). Three of the additional amino acids lengthen helix 4. Such an extension has two structural consequences. First, residues Ala167 and Lys168 are bulged out, compared with their counterparts in Hsp90 (Ala97 and Lys98), and this increases the volume of the second ligand-binding pocket. Importantly, this allows the accommodation of 5′ substituents of the type seen in NECA. Second, this bulge changes the orientation of helix 4 relative to the rest of the protein. Because helices 1 and 5 are reoriented as a consequence of the helix 4 shift, this suggests that these three helices form an integral structural unit. Interestingly, helices 1, 4, and 5 are also the only secondary structural elements of the N-domain that are not tightly associated with the 8-stranded beta sheet that forms the structural core of the protein, suggesting that this subdomain may have the potential to act as a conformational switch. A switch role for helices 4 and 5 has previously been proposed, although not yet observed, for Hsp90 (27).

**Modeling Explains the Unproductive Binding of NECA to Hsp90**—NECA has no detectable binding to cytosolic Hsp90 and is the first high affinity ligand that is selective for a single member of the hsp90 family (38). To understand the basis for this selectivity, we modeled NECA into the ATP binding site of yeast Hsp90, both the apo- and ATP-bound forms, using as the basis for the alignment all residues except those of the helix 1-4-5 subdomain. The validity of this and our other ligand modeling approaches rests, in part, on the observation that the adenine moieties of both NECA and ATP/ADP bind to GRP94 and Hsp90 in an identical manner (Fig. 2, B and C) and that the adenine binding cavity is conserved. The position of the 5′ substituent of the ligand is thus primarily dictated by the adenine backbone. As seen in Fig. 3B, the terminal 5′-methyl group of NECA makes a van der Waals clash with the carbonyl oxygen of yeast Hsp90 Gly121 (corresponding to Gly106 in GRP94) when modeled into yeast Hsp90 (closest approach, 2.4 Å). This clash cannot be relieved by simple rotations about the bonds in the 5′ substituent of NECA. Furthermore, because this clash is with the protein backbone, the structural options for relief are limited to remodelings of the protein that reposition Hsp90 Gly121, a remodeling for which there is no evidence in the 10 Hsp90 N-domain structures solved to date. Thus, the terminal 5′-methyl group is the key discriminating element that imparts selective binding in NECA, and Gly121 is the corresponding protein discriminatory element in cytosolic Hsp90.

Gly121 restricts the binding of phosphorylated nucleotides to GRP94—Although NECA binds to GRP94 with a \( K_p \) of around 200 nM, phosphorylated adenosine nucleotides (e.g., ATP and ADP) bind relatively weakly, with estimated \( K_p \) values in the
millimolar range (38). To understand why phosphorylated nucleosides bind to GRP94 with low affinity, we modeled ADP as seen in the Hsp90/ADP complex into the ligand binding cavity of GRP94, again using the non-helix 1-4-5 subdomain of Hsp90 and GRP94 as the basis for the alignment of the two proteins (Fig. 3C). Examination of this GRP94/ADP model revealed that both the \(\alpha\) and the \(\beta\)-phosphates of the ADP make severe steric clashes with the carbonyl oxygen of Gly\(^{121}\) in GRP94. These clashes could not be resolved by simple rotations of the phosphates or the ribose. Instead, it appears that the same conformation of GRP94 that positions Gly\(^{121}\) in such a way as to allow NECA binding also results in a protein that precludes the binding of AMP, ADP, and ATP by steric interference with \(5^-\)-phosphate groups. This is the inverse of the situation between NECA and Hsp90, where Gly\(^{121}\) (equivalent to GRP94 Gly\(^{196}\)) clashes with the terminal methyl group of NECA and prevents NECA binding. Binding of ATP, ADP, and AMP to GRP94 would therefore appear to require a conformational change in the protein, involving the helix 1-4-5 subdomain, that re-positions Gly\(^{196}\). This predicted requirement for a conformational change in the protein may account for the observed high free energy cost associated with binding ATP to GRP94 (38).

**The Charged Region Forms a Loop**—The three GRP94-ligand complexes whose structures we have determined extend to residue 337 and include the charged region. In all three complexes, no electron density for the charged region residues was observed. Because SDS-PAGE of crystalline GRP94 shows that
the polypeptide chain is intact, and the structures have been determined at high resolution, this indicates that the charged region is either locally disordered or conformationally variable. Surprisingly, however, the residues immediately flanking the charged region, 277–286 (strand 8) and 328–337 (hereafter termed strand 9), associate to form an antiparallel two-stranded β-sheet (Fig. 4B). The association between the two strands is stabilized by bulky side chains from each strand (Trp282 in strand 8, and Trp331 and Trp333 in strand 9) that form an extended series of van der Waals stacking interactions, alternating from one strand or the other (Fig. 4C). Strand 9 thus continues the twisted 8-stranded β-sheet that forms the structurally conserved core of the N-domain. Because strand 8 also marks the transition from the N-domain to the charged linker, the topological consequence of this association between strands 8 and 9 is that the charged region constitutes a loop, and is not, in our structures, a flexible tether between the N- and C-domains. Furthermore, because strand 9 is at the start of the C-domain, the close proximity of the N- and C-domains now suggests a physical mechanism by which the activities of these two domains may be coordinated during client protein maturation.

Comparison with a Yeast Hsp90 Dimer—Because full-length GRP94 and Hsp90 are 42% identical (and overall 70% similar), they are expected to adopt similar tertiary and quaternary conformations. The observation that strands 8 and 9 associate with one another in GRP94, however, is incompatible with the role initially proposed for strand 8 in the structures of the N-domain from yeast Hsp90. In that series of structures, strand 8 (strand h in their nomenclature) forms an antiparallel β-strand interaction with strand 8 from a neighboring molecule in the crystal lattice, thereby forming a symmetrical dimer whose interface is thought to be a potential site of client protein binding (32). A comparison of the GRP94 structure with that of the yeast Hsp90 dimer, however, shows that the strand 8 donated from the neighboring molecule in Hsp90 is superimposable on strand 9 of GRP94 (Fig. 4, A and B). Thus, either the strand 8 dimer as observed in Hsp90 can be formed, or the strand 9 continuation of the β-sheet as observed in GRP94 can be formed, but each possibility precludes the other.

Because neither the yeast nor the human Hsp90 constructs used for crystallization included the charged region, or the putative strand 9, as the GRP94 constructs do, it is possible that the Hsp90 dimer structure is an unintended consequence
of the use of a truncated N-domain for those structural studies. Thus, if a strand 8-strand 9 association does occur in intact Hsp90, it would not have been observed in the shorter constructs used for the yeast and human Hsp90 N-domain crystallizations. As a partial test of this hypothesis, we modeled the amino acid side chains from yeast Hsp90 strands 8 and 9 (residues 203–212 and 263–271) onto the backbone of GRP94 strands 8 and 9 (residues 277–286 and 329–337) as seen in the GRP94 structure (not shown). All of the amino acid side chains from the yeast sequence were easily accommodated in favorable rotamers in the model. Moreover, in addition to preserving the strand 8-strand 9 hydrogen bonds, a favorable van der Waals interaction was observed between Gln570 and Ile587 of the Hsp90 model. This supports the suggestion that a strand 8-strand 9 interaction may also occur in cytoplasmic Hsp90s.

Because the 40-amino acid charged region was disordered in the crystal structure of the N-domain of GRP94 and is long enough to potentially span a considerable distance in the crystal lattice, we could not exclude the possibility that strand 9 came from another protomer in the crystal lattice, thus forming a mixed dimer crystallization artifact. To investigate this possibility, a GRP94 N-domain construct (69–337,Δ40) was made where the charged residues 287–326 were replaced by four glycines, thus shortening the charged domain internal loop by a net of 36 amino acids. There is a biological precedent to accompany the structural evidence presented here for this deletion, because both mitochondrial TRAP-1 (2 residues) and bacterial HtpG (~10 residues) have short or non-existent charged linker domains. Purified GRP94-(69–337,Δ40) elutes as a monomer on gel filtration and binds NECA in the same manner as the parent molecule.

We solved the structure of GRP94-(69–337,Δ40) in the NECA-bound form, and the structure of this mutant supports the original observation that strands 8 and 9 are associated. First, strand 9 again forms a beta sheet with strand 8, just as it does in the parent molecule, despite the deletion of 36 of the 40 intervening amino acids. Second, although the four glycine linker residues are disordered in the crystal structure of the mutant protein, the packing of the proteins in the crystal lattice places the symmetry related molecules too far apart for the four glycines to form a different molecule. Therefore, the strand 8/9 interaction in GRP94-(69–337,Δ40) must be intramolecular. Third, the identical behavior of the wild-type and Δ40 mutants in NECA binding and on gel filtration chromatography (not shown) supports the crystallographic observation that strands 8 and 9 of GRP94 associate with one another. Finally, it has been observed that GRP94 N-domain truncation mutants that contain a complete strand 8 but lack all or even part of strand 9, are insoluble.2 This suggests that strand 8 requires strand 9 for stability, and this can easily be explained by the extensive complementary interactions between the non-polar residues on strands 8 and 9 as seen in the GRP94 N-domain structure (Fig. 4C). Together, this evidence supports the conclusion that the strand 8/9 interaction is intramolecular and is not an artifact of the crystallographic analysis.

**DISCUSSION**

We report here the structure of the N-terminal and charged domains of the hsp90 chaperone GRP94 in complex with several high affinity ligands and show that a subdomain of GRP94 adopts a different conformation from the equivalent region of cytoplasmic Hsp90. The structure explains the origin of specific NECA binding to GRP94, suggests the presence of a ligand-responsive conformational switch, and reveals a previously unknown means of association between the N- and C-terminal domains of the complete chaperone. These discoveries have several implications, which we discuss below.

A characteristic feature of the hsp90 chaperones is the formation of highly active multimeric oligomers in vitro in response to heat shock and certain chemical stimuli (51–54). Although hsp90 proteins have a strong dimerization interface within their C-domains, the intact hsp90 dimer must also expose at least one additional dimerization surface if it is to form multimeric aggregates. This suggests the existence of a transiently stable second dimerization interface that would be protected under normal conditions but would be exposed upon heat shock or other forms of stress.

A second dimerization interface in any hsp90 other than the primary one in the C-domain has not yet been convincingly identified, nor do the GRP94 N-domain structures presented here associate in the crystal lattice in a manner that readily suggests a biologically relevant dimer interface. However, attempts to reconcile the result shown here that strands 8 and 9 of GRP94 associate with one another, with the observations of the Pearl group that the yeast Hsp90 N-domains form crystallographic dimers via strand 8 (32) leads us to suggest that strand 8, which, under heat stress, may become exposed by dissociating from strand 9, could be a candidate for the second dimerization interface. Experiments are currently underway to test this hypothesis.

An alternative dimer interface, other than the one involving strand 8, has recently been proposed for yeast Hsp90 (27, 28). Like the strand 8 interaction suggested originally (32), this newly proposed interface also spans a crystallographic dyad and resembles the dimer interface seen in GyrB and MutL, other members of the GHKL family. It also places the ATP binding cavities from the two protomers in opposition to one another, thus potentially allowing for signaling of ligand occupancy between protomers. Although this model is attractive on grounds of structural similarity, a similar interface, crystallographic or otherwise, has not been observed for GRP94 in the four structures we have solved (in multiple space groups), nor was a similar interface observed in the highly homologous human Hsp90 N-domain crystal structure (16, 30). We are therefore not able to confirm the validity of this alternative N-domain dimer interface.

Although competitive inhibitors of ATP binding inhibit the activity of both Hsp90 and GRP94, previous structural studies have yet to provide insight into the mechanism by which ligands modulate Hsp90 or GRP94 chaperone activity. In particular, the structures of the apo-, ATP/ADP-, radicicol-, and geldanamycin-bound Hsp90 complexes are all identical. This suggests that cytoplasmic Hsp90 chaperone activity may be modulated by factors other than conformational changes within the N-domain of the chaperone itself. In the structures presented here, we have shown that the conformation of GRP94 in complex with several non-phosphorylated ligands differs from that of cytosolic Hsp90. Importantly, however, these comparisons highlight the fact that this particular conformation of GRP94 is incompatible with the binding of ATP, ADP, or AMP. Because adenine nucleotides do in fact bind to GRP94, this strongly implies that the conformation of the helix 1-4-5 subdomain of GRP94 in the AMP-, ADP-, and ATP-bound forms will differ from that observed in the NECA-, radicicol-, and 2ClddA-bound forms, and would therefore represent a ligand-dependent conformational switch. A ligand-dependent conformational change in GRP94 has been proposed based on cryptophan fluorescence experiments (38) and is also supported by the observation that GRP94 oligomerization behavior

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2 J. Baker and C. V. Nicchitta, unpublished observations.
changes in response to different ligands.3 Whether this putative helix 1-4-5 switch is unique to GRP94 or common to all hsp90s is as yet unclear. In this regard, it is worth noting that the molecular switch we have tentatively identified differs from the “lid” switch proposed, but not yet observed, for Hsp90 (27). This proposed lid consists of helices 4 and 5 and the loop connecting them (see Fig. 3A). It was suggested that these helices pivot around residues Gly100 and Gly213 (equivalent to GRP94 Gly170 and Gly186) and cover the ligand binding cavity in response to ligand occupancy. However, in GRP94 we observe that helices 1, 4, and 5 appear to act as an integral structural unit, which would be incompatible with a large independent movement of helices 4 and 5. The mechanism and events leading to lid closure, if it does occur, may also be different for Hsp90 and GRP94. Further experiments will be needed to explore these possibilities.

The current model for Hsp90 regulation envisions ATP binding and hydrolysis, in concert with co-chaperone binding, as key modulators of chaperone activity. GRP94 differs from its cytoplasmic paralog in that ATP hydrolysis above background levels has not been detected. Co-chaperones for GRP94, including those that might stimulate ATP hydrolysis in the way that Aha1 does for human Hsp90 (34), have also not been identified. This may indicate that Hsp90 and GRP94 are governed by different regulatory mechanisms and that the role of ATP in the regulation of the different hsp90 chaperones also may be different. Given these differences, it is possible, for example, that the role of the regulatory ligand in GRP94 may be to induce a particular conformation in the chaperone that leads to gain or loss of activity. If ATP is the regulatory ligand for GRP94, this may explain why the binding constant for ATP (∼mM) is close to the physiological concentration, because variation in the cellular level of ATP could lead to changes in GRP94 occupancy. This would contrast with Hsp90, where ATP binding and hydrolysis are required to regulate the interaction of co-chaperones such as p23 with Hsp90 (30). A first test of this hypothesis would be the experimental demonstration of a ligand-dependent conformational switch in the N-domain of GRP94, and these experiments are in progress.

Finally, we have shown here the structural basis for the binding of small molecules to GRP94, a ligand that is selective for GRP94, and does not bind to Hsp90. Because GRP94 is required for the maturation of proteins involved in innate immunity (10), such ligands and the identification of a novel binding pocket adjacent to the adenine binding cavity in GRP94 open up the possibility of designing selective high affinity molecules that may be pharmacologically relevant in the treatment of septic shock, coronary thrombosis, stroke, and other diseases.

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