Structural and functional analysis of GRP94 in the closed state reveals an essential role for the pre-N domain and a potential client-binding site

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Summary

Hsp90 chaperones undergo ATP-driven conformational changes during the maturation of client proteins, populating a “closed” state upon ATP binding in which the N-terminal domains of the homodimer form a second inter-protomer dimer interface. A structure of GRP94, the endoplasmic reticulum hsp90, in a closed conformation has not been described, and the determinants that regulate closure are not well understood. Here, we determined the 2.6 Å structure of AMPPNP-bound GRP94 in the closed dimer conformation.

The structure includes the pre-N domain, a region preceding the N-terminal domain that is highly conserved in GRP94, but not in other hsp90s. We show that the GRP94 pre-N domain is essential for client maturation, and identify the pre-N domain as an important regulator of ATPase rates and dimer closure. The structure also reveals a GRP94:polypeptide interaction that partially mimics a client-bound state. The results provide structural insight into the ATP-dependent client maturation process of GRP94.

eTOC Blurb
Huck et al. determine the structure of the ER hsp90 chaperone GRP94 and show that it adopts a closed dimer conformation. The non-conserved pre-N terminal domain is shown to be essential for GRP94 client maturation. A captured polypeptide fragment in the structure mimics a bound client.

Keywords
GRP94; Hsp90; chaperone; TRAP1

Introduction

GRP94 is the endoplasmic reticulum (ER) resident member of the hsp90 family, a highly conserved and ubiquitous family of ATP-dependent molecular chaperones. Hsp90s govern the maintenance and activation of many essential proteins (Marzec et al., 2012; Whitesell and Lindquist, 2005). In the ER, GRP94 has been shown to be essential for the maturation of membrane resident and secreted protein clients (Hong et al., 2017; Liu et al., 2013; Ostrovsky et al., 2009; Randow and Seed, 2001; Staron et al., 2010; Weekes et al., 2012; Yang et al., 2007). Elevated expression of GRP94 in cancers is associated with aggressive behavior and poor prognosis (Lee, 2014; Zheng et al., 2008). As the oncogenic roles of the ER hsp90 come into focus (Hua et al., 2013; Patel et al., 2013), the inhibition of GRP94 offers the prospect of disrupting several key cancer pathways including cell proliferation, immunosuppression, and inflammation (Ansa-Addo et al., 2016; Wu et al., 2015).

Higher eukaryotes contain four hsp90 paralogs. In addition to GRP94, Hsp90α and β are cytosolic, and TRAP1 is mitochondrial (Johnson, 2012). (Here, hsp90 refers to the chaperone family, while Hsp90 refers to the cytoplasmic paralog.) All hsp90s are homodimers and contain three major domains: the N-terminal (N), middle (M), and C-terminal (C) domains. The N domain is the site of ATP binding, the M domain is involved in ATP hydrolysis and client protein recognition, and the C domain is the site of constitutive dimerization (Prodromou, 2016). Protein folding by hsp90s depends on conformational changes to the
chaperone driven by cycles of ATP binding and hydrolysis, as well as by interactions with client proteins, cochaperones, and the Hsp70 system (Karagoz and Rudiger, 2015). Our understanding of the integration of the chaperone, cochaperone, and client pathways has been broadened by crystal structures of chaperone-cochaperone complexes (Ali et al., 2006), by NMR studies of chaperone-client complexes (Karagoz et al., 2014; Lorenz et al., 2014) and by a cryo-EM study of a complex between human Hsp90, the cochaperone Cdc37, and the client kinase Cdk4 (Verba et al., 2016). In this later assembly, an unfolded segment of the client protein is clamped in a channel formed between the luminal faces of the opposing protomers of the dimer. It is not known if this client capture mechanism reflects a general mechanism for other clients and other paralogs.

Despite having high sequence homology, hsp90 paralogs hydrolyze ATP at significantly different rates (Dollins et al., 2007; Krukenberg et al., 2011). Structures of nucleotide-bound cytosolic Hsp90 and GRP94 have highlighted mechanistic differences in the ATPase cycle. In complex with the cochaperone p23, yeast Hsp90 adopts a “closed dimer” conformation where the helix 4/5 “lid” of the N-domain closes over the ATP-binding pocket and strand 1 and helix 1 of the two N domains swap between protomers to form a second dimer interface (Ali et al., 2006). The catalytic residues from the N and M domains are aligned with the γ-phosphate of the bound AMPPNP. In contrast, the structure of an equivalent GRP94 construct that included the lid, strand 1 and helix 1, bound to AMPPNP, revealed a catalytically incompetent “twisted V” conformation characterized by an extended open lid and separated, rotated, N domains (Dollins et al., 2007). Correlating the unexpected crystal state of this GRP94 construct with the finding that ATP hydrolysis is essential for GRP94 client maturation (Randow and Seed, 2001) suggests that additional factors may be required to stabilize the catalytically active conformation.

The fact that higher eukaryotes maintain an abundant specialized hsp90 in the ER suggests that GRP94 is uniquely evolved for its role in the ER. In addition to differences in structure and ATP hydrolysis rates, GRP94 lacks the retinue of cochaperones characteristic of cytoplasmic Hsp90. In addition, while some GRP94 domains are able to functionally substitute for their yeast Hsp90 counterparts, the equivalent yeast Hsp90 domains fail to replace their GRP94 counterparts in client maturation assays (Maharaj et al., 2016). These observations suggest that regions of divergence between the chaperones may lead to functional differences that are key to GRP94’s adaptation to the ER. One such region, the pre-N domain, is located immediately upstream of strand 1 of the N domain and varies substantially among hsp90s (Partridge et al., 2015). GRP94 contains the longest pre-N domain in the hsp90 family, consisting of residues 22–72 of the mature protein (Fig. S1). The GRP94 pre-N domain suppresses the rate of ATP hydrolysis (Dollins et al., 2007), but how it does so and its role in the function of GRP94 is not known. Interestingly, the TRAP1 pre-N domain also suppresses the rate of ATP hydrolysis (Lavery et al., 2014). Recent crystal structures of TRAP1 have shown that the TRAP1 pre-N domain forms “straps” that extend the interactions formed by the two strands 1 as they cross between the opposing N domains in the full length dimer (Lavery et al., 2014), or wrap around their own N-domains in cis in structures of the non-dimeric NM domain (Lee et al., 2015b). It is not known if the GRP94 pre-N domain serves a similar structural role. The construct used for an earlier near-full length GRP94 crystal structure (Dollins et al., 2007) began with strand 1 but did not...
contain the pre-N domain and thus could not be used to address the structural and functional properties of this domain.

To better understand the role of the GRP94 pre-N domain, we determined the crystal structure of a GRP94 construct that includes the pre-N domain, in complex with AMPPNP. Our structure reveals that GRP94 adopts the closed dimer conformation that is poised for ATP hydrolysis. We show that the GRP94 pre-N domain crosses to the opposite protomer as part of the N domain dimerization but makes only weak interactions in doing so. We also present biochemical data that shows that a short region within the pre-N domain regulates the rates of both ATP hydrolysis and N domain dimerization. Going beyond its regulation of ATPase activity, we now identify the functional importance of the pre-N region and show that virtually the entire pre-N domain is required for client maturation. Finally, the structure we determined reveals an unexpected interaction between crystallographically related GRP94 dimers that partially mimics a client-chaperone interaction.

**Results**

**GRP94 containing the pre-N domain forms a closed dimer**

We solved the crystal structure of a near-full length GRP94 (residues 48–754Δ287–327) containing a portion of the pre-N domain (residues 48–72) and lacking the charged linker and the C-terminal extension, bound to AMPPNP. The model was refined against data collected to 2.62 Å (Table 1).

The structure contains two GRP94 protomers arranged as a homodimer (Fig. 1A). In contrast to the earlier GRP94 structure (Dollins et al., 2007), GRP94 with the pre-N domain adopts a closed dimer conformation. In addition to the primary dimer interface between the opposed C-terminal domains, a second homodimer interface is now present in the same dimer between the two opposed N-terminal domains. This additional dimer interface is formed by the interaction between the N1 helices of the opposed N-terminal domains, and is further stabilized by a swap across the dimer interface between strands N1 (residues 73–78) of the opposing protomers (Fig. 1B).

The GRP94 closed dimer is symmetric, with an RMSD between the Cα atoms of the two protomers of 0.58 Å. This distinguishes GRP94 from the asymmetric arrangement of TRAP1, where the two protomers of the dimer differ in the configuration of their middle and C-terminal domains and have an RMSD of 3.34 Å between Cα atoms. The GRP94 closed dimer is visualized in the catalytically competent state. Arg448 from the M domain, which is required for ATP hydrolysis in GRP94 (Dollins et al., 2007), is within hydrogen bonding distance of the γ-phosphate of the ATP and is poised to stabilize the leaving group (Fig. 1C).

In addition, the lid is closed over the ATP-binding pocket and protects the bound nucleotide from exchange (Fig. 1C). The disposition of the lid in the ATP cycle of GRP94 has been unclear (Dollins et al., 2007; Frey et al., 2007). The GRP94 lid has a unique 5 amino acid insertion not found in other hsp90 paralogs, and structures of the isolated GRP94 N domain showed that the lid moves from the apo “open” state to an idiosyncratic “extended open” conformation when nucleotides are bound, exposing both the lid and the bound nucleotide to solution (Fig. S2) (Immormino et al., 2004). The lid conformation revealed in the closed
dimer structure conclusively establishes that the GRP94 lid closes over the bound ATP to complete the catalytic cycle.

**The pre-N domain makes sparse interactions with the N domain**

The pre-N domain precedes strand N1 and contains two ordered regions, hereafter referred to as the proximal (residues 63–72) and distal (residues 48–62) pre-N regions. The two proximal pre-N regions are both ordered and make similar interactions with their opposed N domains (Fig. 1B,D). Only one of the two distal pre-N regions is ordered, however, and, as will be discussed later, it interacts with a symmetry-related GRP94 dimer.

The proximal GRP94 pre-N residues wrap over and behind the N-terminal domain of the opposing protomer of the closed dimer but make few connections with the N domain. Residues 66–72 of the proximal pre-N cross strand N6 of the opposite protomer at an unfavorable ~50° angle and make only one connecting hydrogen bond (Fig. 2A). The pre-N domain then makes a 90° turn at Ile66 and continues parallel to strand N7 of the N-domain β-sheet. This turn is guided by the side chain of Arg237, which makes the lone additional pre-N:N-domain hydrogen bond to the backbone carbonyl oxygen of Gln65 (Fig. 2A).

Residues of the proximal pre-N make few van der Waals contacts with the N domain, packing only loosely against the back face of the N domain β-sheet and allowing for significant voids. The two proximal pre-N regions enlarge the second dimer interface only slightly, adding just 1760 Å² of solvent excluded surface area compared to a dimer without the pre-N.

The GRP94 proximal pre-Ns follow a different path and make looser connections to the N-domain compared to the equivalent region in TRAP1. The pre-N of that paralog makes extensive hydrogen bonding interactions with strand N6 and a series of van der Waals and hydrogen bonding contacts as it continues straight down the back of the β-sheet core of the opposing N domain (Fig. 2B,C). Reflecting this tight interaction, the TRAP1 pre-N “straps” add 3125 Å² of solvent excluded surface area to the TRAP1 dimer.

The path of the TRAP1 pre-N is incompatible with the GRP94 N domain. This is due to predicted clashes with a 3 amino acid insertion in the loop between strands N6 and N7, and with Trp333 of the “tryptophan zipper” that stabilizes the strand N8/N9 interaction (Soldano et al., 2003) and charged linker (Fig. 2D). The presence of an enlarged loop between strands N6 and N7, a charged linker, a Trp-zipper, and extensive strands N8 and N9 all distinguish the N-terminal domain of GRP94 from that of TRAP1, and explains why the paths of the pre-N domains from the two paralogs differ. These differences may indicate distinct roles for the pre-N domains of each paralog.

**GRP94 client maturation requires the pre-N domain**

The role of the pre-N domain in client maturation is unknown and has never been tested in any hsp90 paralog. The GRP94 pre-N domain is highly conserved beyond the first 7 residues and is distinct from that of all other hsp90 paralogs (Fig. S1). Client proteins, such as TLRs and integrins, are strictly dependent on GRP94 for proper activation and localization to the cell surface (Randow and Seed, 2001; Yang et al., 2007). To probe the functional relevance
of the pre-N domain, we tested the ability of truncated GRP94 constructs to mature client proteins \textit{in vivo}.

Genes coding for GRP94 proteins bearing truncations of the pre-N domain were cloned into retroviral vectors (Fig. 3A). Virions expressing these constructs were transduced into a GRP94\textsuperscript{null} pre-B cell line, E4.126, which is viable in the absence of GRP94, but unable to mature GRP94 clients (Randow and Seed, 2001). The surface expression of endogenously encoded mature clients reflects a functional virally-transduced GRP94 and was quantitatively measured using flow cytometry.

Remarkably, as summarized in Fig. 3A and shown in Fig. S3A, most pre-N truncation mutants failed to support maturation of the CD11a, CD49d, and TLR2 clients. Only wild-type GRP94 (MZG1) or GRP94 mutants harboring deletions or replacements in the first five residues, which are less conserved, were functional. Intracellular staining for GRP94 demonstrated that each construct tested was expressed at levels similar to wild type (Fig. S3A). The chaperone function of GRP94 thus has a strict requirement for the pre-N domain \textit{in vivo}.

**A short region of the pre-N domain regulates the GRP94 ATPase rate and dimer closure**

Complete truncation of the GRP94 pre-N domain results in a 5-fold increase in the rate of ATPase activity compared to wild-type (Fig. 3B) (Dollins et al., 2007). Given the loose association between the proximal pre-N and the opposing N domain described above, we made a series of pre-N deletions to understand how the pre-N domain suppresses the rate of ATP hydrolysis. As seen in Fig. 3B, GRP94 molecules with pre-N domains starting at residues 38, 48, and 58 exhibit low, wild-type like ATP hydrolysis rates. By contrast, GRP94 molecules that start at residue 63, the first ordered residue of the proximal pre-N domain, possess an intermediate level of hydrolysis activity. Control experiments showed that the charged linker or the presence of the His-tag did not affect the ATPase activity of the protein (Fig. S3B).

ATP hydrolysis requires that GRP94 occupy the closed dimer state. To characterize the transitions between GRP94 conformational states, we used FRET to monitor the relative arrangements of the N and M domains of the chaperone as a function of bound nucleotide. We compared the signal to that of yeast Hsp90 using equivalent donor and acceptor sites. Donor- and acceptor-labeled proteins were mixed to form equilibrated populations of GRP94 or yeast Hsp90 donor/acceptor heterodimers. AMPPNP was added to the equilibrated donor/acceptor heterodimers to initiate the transition to the closed conformation. As seen in Fig. 3C, full truncation of the pre-N domain (73 start) accelerates dimer closure ~7-fold compared to full-length GRP94. Truncation of residues 22–57 (58 start) results in a suppressed closure rate that nearly matches that of full-length GRP94. The FRET data correlates with the ATPase rates, suggesting that dimer closure governs the rate of hydrolysis (Fig. 3D). Together these data implicate residues 58–62 as a key regulatory region governing the structural transitions to closure. Residues 58–62 are disordered in the GRP94 crystal structure, however, suggesting that they regulate the rate of ATP hydrolysis not in the closed dimer state but via conformations that precede the closed dimer in the ATPase cycle.

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The distal pre-N domain is captured by a neighboring dimer to mimic a bound substrate

Residues 49–60 of the distal portion of one of the two the pre-N domains are bound into a channel formed between two loops in the M domain (residues 394–407) and the GRP94 “client-binding domain” (CBD, residues 652–678) (Wu et al., 2012) of a symmetry-related dimer (Fig. 4A, Fig. S4A,B). Remarkably, the channel-bound distal pre-N domain from the GRP94 structure is nearly identical to the partially unfolded region of the client protein Cdk4 that is clamped into the equivalent channel of human Hsp90β, as seen in the cryo-EM structure of the Hsp90β:Cdc37:Cdk4 complex (Verba et al., 2016). Alignment of GRP94 and Hsp90 from these two structures shows that, despite having different amino acid sequences, the distal pre-N domain backbone is superimposable with the unfolded portion of Cdk4 (Fig. 4B). Because of the similarity of these two interactions, we propose that the distal portion of the pre-N domain is a partial mimetic of the interaction between an unfolded substrate and GRP94.

There is an increase in crystallographic order in regions around the lumen-bound pre-N domain compared to other hsp90 crystal structures. In the GRP94 closed dimer, M domain loop residues 394–407 are fully ordered and close over the inserted distal pre-N domain via contacts between Gly402/Ser403 pairs from the opposing protomers (Fig. 4A). These loops are disordered in the GRP94 twisted V structure and only partially ordered in the full length TRAP1 and yeast Hsp90 structures, suggesting an inherent flexibility to this loop in the absence of a substrate or substrate mimetic.

The GRP94 luminal channel is formed by Phe398, Asp399, Glu400, Tyr401, Lys428, Ile497, Glu498, Tyr575, Met662, Gln665, Ala666, Tyr667, and Gln668 from both protomers of the GRP94 dimer. Despite fully encircling the pre-N domain fragment, however, there are few contacts between the chaperone and the proposed substrate mimetic. As seen in Fig. 4D, Gln665 residues from the CBD of both protomers hydrogen bond to the backbone of the distal pre-N domain and provide the only direct contacts. Mutation of Gln665 to alanine does not disrupt GRP94 client maturation however (Fig. S3A), suggesting that these contacts are not essential to the loading or positioning of client substrates.

The lack of interactions between the captured peptide and the chaperone reflects the size of the luminal opening, which, at 7–8 Å horizontally and 11–12 Å vertically, is large enough to accommodate a variety of polypeptides in an extended conformation but is too small to fit polypeptides folded into α-helices or other secondary structures. In keeping with the loose fit of the inserted peptide, replacement of the M domain loop residues (395–406) with 4 glycines did not prevent maturation of GRP94 client proteins (Fig. S3A).

Discussion

The GRP94 structure determined here shows that the ER paralog adopts a closed dimer conformation in response to nucleotide binding, and supports a unified mechanism for ATP hydrolysis among hsp90 chaperones. The proximal regions of the two pre-N domains swap between protomers but, surprisingly, add little to the N domain dimer interface. Truncation analysis of the pre-N domain points to an essential role for the entire region in client maturation, and identifies an internal region that regulates dimer closure and ATPase rates.
Finally, the structure reveals an adventitious insertion of the distal pre-N domain into a channel formed between the M and C domains of a neighboring dimer, in apparent partial mimicry of a bound client.

Although the closed-lid, closed dimer structure now places GRP94 along the same ATP-driven conformational pathway as other hsp90 paralogs, earlier structural and biochemical data have supported a model in which GRP94 largely populates an open-like conformation in both the presence and absence of nucleotides (Dollins et al., 2007; Frey et al., 2007; Krukenberg et al., 2009). These discrepancies can be reconciled in part by considering the slow rate of conformational transitions observed for GRP94. The FRET analysis shows that at 37 °C, the rate of closed dimer formation for GRP94 is over two orders of magnitude slower than for yeast Hsp90 (Fig. 3C). FRET data also show that there is a strong temperature dependence on the transition to the catalytically active state for GRP94, with the rate decreasing by at least two orders of magnitude when the temperature is lowered from 37 °C to 30 °C (Fig. 3C). This finding may explain the apparent nucleotide-insensitivity of GRP94 observed by SAXS (Krukenberg et al., 2009). Those experiments were carried out at 25 °C, a temperature that may have been too low to allow for ATP-driven redistribution of the GRP94 conformational states.

The slow kinetics of closed dimer formation we report here may also explain the apparent failure of GRP94 to protect bound ATP from exchange during hydrolysis (Frey et al., 2007). In those experiments, an unlabeled ATP chase was introduced 3 minutes after the hydrolysis reaction was started. The FRET data we have presented shows that the transition to the closed dimer - and closed lid - state takes considerably longer than this, and may therefore account for the loss of nucleotide protection observed in the pulse chase experiment.

Together, these data serve to highlight the high barrier to N dimer closure in GRP94. In part this is due to the unique GRP94 N domain and its idiosyncratic lid, which, by virtue of its propensity to adopt the extended open conformation upon nucleotide binding, presents one barrier to closure (Dollins et al., 2007). In addition, we now report that the 58–62 region of the pre-N domain provides additional suppression of the closing transition. This is unlikely to be an effect of the pre-N domain acting on the closed dimer state, since the structure shows that the contacts between the proximal pre-N and the N domain are sparse, especially when compared to the tight association between the pre-N and N domains of TRAP1. Instead, it is likely that the regulatory effects of the pre-N domain are manifested prior to the formation of the closed dimer state. Thus, for example, it is possible that the GRP94 pre-N domain forms a tight interaction in cis with its own N domain while in an open or twisted V state. Since closed dimer formation requires that the pre-N domain be free to interact with the opposite protomer of the dimer, suppression of closed dimer formation would come about by the need to “peel” off the pre-N from its resting place. This concept has been outlined previously (Partridge et al., 2015) and a pre-N:N domain interaction in cis that recapitulates the association in trans observed in the full length structure has been demonstrated in the crystal structure of a TRAP1 NM fragment (Lee et al., 2015a). Because a tight interaction between the pre-N and GRP94 N domain was not observed in the closed dimer structure, however, it is possible that the regulatory interactions between the pre-N and the rest of the GRP94 molecule are unavailable in the closed dimer conformation.
Interestingly, GRP94s from plants lack the equivalent of residues 58–65 in their pre-N domains (Fig. S1), and the ATPase rate of Arabidopsis GRP94 is 30-fold greater than that of metazoan GRP94 (Chong et al., 2015). Although part of this faster rate may be due to differences in the N-terminal domain lid, the correlation between the lack of the 58–63 pre-N regulatory region in Arabidopsis GRP94 and the fast ATPase rate is unlikely to be a coincidence.

The role of the GRP94 pre-N domain in governing ATP hydrolysis and dimer closure highlights differences between the modes of regulation between the organellar and cytoplasmic hsp90 paralogs. For Hsp90, which lacks a pre-N domain, numerous cochaperones including Hop/Sti1, Cdc37, p23, and Aha1 stabilize the open or closed dimer state and inhibit or accelerate ATP hydrolysis (Rohl et al., 2013). ER and mitochondrial equivalents of these cochaperones are not present, but both GRP94 and Trap1 contain a significant pre-N domain. It is possible that, in the pared-down chaperoning world of the organellar compartments, the complexity of cochaperone regulation is replaced by intrinsic regulation via unique chaperone extensions, as in GRP94, or other structural adaptations such as the asymmetry found in Trap1 (Elnatan et al., 2017; Lavery et al., 2014).

Our structure of GRP94 in the catalytically active conformation has also unexpectedly revealed a potential mode of client binding to GRP94. As a result of crystal packing, the distal pre-N domain of one of the two GRP94 protomers is threaded through the luminal channel of a neighboring dimer in the crystal lattice. Several aspects of this interaction are reminiscent of the bona fide Hsp90:client interaction seen in the recent cryo-EM structure of an Hsp90:Cdc37:Cdk4 complex (Verba et al., 2016). Both chaperones are in the closed dimer conformation, implicating ATP binding in the substrate acquisition step and ATP hydrolysis as a client release mechanism. The path and conformation of the threaded pre-N peptide is also similar to that taken by the unfolded portion of Cdk4, which threads through the equivalent luminal space of Hsp90.

The partial client mimetic:GRP94 interaction we have observed differs from chaperone-substrate complexes seen in previously described complexes between Hsp90β and tau (Karagoz et al., 2014) and yeast Hsp90 and the glucocorticoid receptor ligand binding domain (GRlbd) (Lorenz et al., 2014). Unlike the closed dimer GRP94 structure seen here, the Hsp90β:tau complex is modeled in the open chaperone conformation. NMR chemical shift perturbation analysis implicates residues along the inner face of the N and middle domains of the Hsp90β as interacting with the bound tau substrate. Of the identified residues, only Hsp90β Phe343 (GRP94 Phe398) overlaps with the luminal channel residues through which the GRP94 pre-N peptide threads. Similarly, the yeast Hsp90:GRlbd study identified 40 substrate-interacting residues by chemical shift perturbation, of which only 2, His430 and Glu431 (GRP94 Ile497 and Glu498) overlap with the GRP94 lumenal channel. The side chains of GRP94 do not appear to bind specifically to the captured peptide in our structure, suggesting that substrate recognition is likely to be mediated by interactions that would involve other surfaces of the chaperone and other parts of an intact client. Yet to be determined is the role of accessory factors and client unfolding in the formation of a potential GRP94:client assembly. We have shown here that the GRP94 pre-N domain is...
required for client maturation. The mechanism by which this occurs is unknown. Because the pre-N domain does not appear to contribute to the stability of the closed dimer, an alternate possibility is that the pre-N domain acts as an intrinsic recruitment factor for the ER resident clients in place of the cytosolic cochaperones. Due to the highly charged nature of the pre-N domain, it has long been assumed that the main role of this domain was to mediate the calcium binding activity of GRP94 (Koch et al., 1986; Van et al., 1989). Yet calcium has no effect on ATP hydrolysis or the overall conformation of the chaperone (Dollins et al., 2007; Krukenberg et al., 2009; Li and Srivastava, 1993) and is therefore unlikely to play a significant role in the chaperoning activities of GRP94. Instead, the data presented here show a surprising dependence of client maturation on the GRP94 pre-N domain. The GRP94 pre-N domain is highly conserved, it is longer than any other hsp90 pre-N domains, and follows a unique structural trajectory compared to the next longest pre-N domain seen in TRAP1. Together these observations support the idea that the pre-N domain may serve as an intrinsic means of client recruitment or recognition, either through direct interactions with clients or other ER chaperone systems such as BiP.

The structure of the Hsp90:Cdc37:Cdk4 complex identified a novel Cdk4 conformation in which Hsp90 clamps around an unfolded β sheet, separating the two lobes of the kinase (Fig. S4C). In contrast to Hsp90 clients, which bear little structural resemblance to each other, many GRP94 clients contain common structural motifs, such as the β-propeller, the leucine-rich repeat, or repeated small domains (Weekes et al., 2012). This suggests that GRP94 clients may have a limited number of modes of interaction with the chaperone. Moreover, by analogy with the split kinase model of interaction, the high β sheet content of many of these motifs may predispose these clients to unfold if they bind in the channel of GRP94.

The observation of a potential peptide-binding channel in GRP94 lends support to the proposal that GRP94 interacts with the peptide repertoire of the ER as part of the MHC peptide presentation machinery (Li and Srivastava, 1993; Srivastava, 2002). A peptide fragment derived from the αI domain (AID) of integrin αL has also been reported to suppress integrin maturation (Hong et al., 2013). It is possible that the target of these peptides is the same channel observed to bind the pre-N domain in our structure.

In summary, we have shown that GRP94 adopts the catalytically active closed conformation characteristic of the hsp90 chaperone family. Our structure reveals that the proximal pre-N domains swap between protomers, but make few interactions that add to the stability of the closed dimer state. We have shown that virtually the entire pre-N domain is required for GRP94 client maturation, a remarkable finding that points to a critical and perhaps specialized role for this longest- and best-conserved pre-N region among hsp90 family members. We have also identified a short region joining the distal and proximal portions of the pre-N domain that contributes to the regulation of the conformational kinetics of GRP94, suggesting that the pre-N domain fulfills a number of roles for this chaperone. Finally, our structure has unexpectedly revealed a peptide bound to a channel in the lumen of the GRP94 dimer that interacts with a functionally required region of the structure and that bears a strong resemblance to the portion of a kinase bound to a similar region of Hsp90. This points to a possible conserved mechanism for client:chaperone interactions among the hsp90
family members, and provides a starting point for future investigations of how GRP94 interacts with *(bona fide)* client proteins.

**Experimental Procedures**

**Protein Expression and Purification**

Canine GRP94 was expressed in E.coli an N- or C-terminal His-tag fusions and purified by Ni-affinity, anion exchange, and size-exclusion chromatography.

**Crystallization**

GRP94(48–754Δ287–327) at 30 mg/mL was co-crystallized at 23 °C with 4-fold molar excess of AMPPNP by vapor diffusion. Crystallization reservoirs contained 0.8M-1.2M NH₄Cl and 8.5–11% PEG 3350. Crystals reached full size in 1–2 days.

**X-ray Data Collection**

Fresh crystals (<2–3 days after set-up) were gradually equilibrated in cryo-protection solutions consisting of 0.2 M NH₄Cl, 10–15% PEG 3350, and 10–15% ethylene glycol before flash freezing in liquid nitrogen. X-ray data (jh22a5b) was collected at Advanced Photon Source (APS) beamline 23-IDB at 100K using a MARCCD and processed using XDS.

**Structure Solution and Refinement**

GRP94 bound to AMPPNP was solved by molecular replacement. Model building was completed in COOT (*Emsley et al., 2010*) and refined using PHENIX (*Adams et al., 2010*). Structure validation was performed using MolProbity and molecular graphics were created using PyMol.

**Flow Cytometry**

Transduced cells were incubated with biotin-conjugated primary antibody at 4°C for 30 minutes, followed by incubation with a streptavidin-conjugated Allophycocyanin (APC) fluorescent antibody. Before instrumentation, 7-aminoactinomycin D (7-AAD) was added to stain and exclude dead cells. For intracellular staining of GRP94, cells were fixed in 4% paraformaldehyde, permeabilized in ice-cold methanol, and stained with antibody. Cells were acquired with a FACSVerse flow cytometer (BD Biosciences). Data analysis was done using FlowJo software.

**ATP Hydrolysis Assay**

ATP hydrolysis experiments were performed using the PiPer Phosphate assay kit (Thermo Fisher). The data were plotted using Prism software and fit with the Michaelis-Menten equation.

**FRET experiments**

GRP94 was site-specifically labeled with donor or acceptor maleimide dyes (Alexa Fluor 555 and 647, respectively) at indicated sites. Donor and acceptor labeled proteins were
mixed at a 1:1 ratio at 37°C for 30 minutes to a final concentration of 250 nM. Experiments were performed at 37°C with excitation/emission wavelengths of 532/667 nm. Closure was initiated by addition of concentrated AMPPNP to a final concentration of 2 mM. Closure rates were determined by fitting data with a one-phase exponential decay function in Prism.

Supplementary Material
Refer to Web version on PubMed Central for supplementary material.

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Highlights

- AMPPNP bound GRP94 adopts the closed dimer conformation
- The GRP94 pre-N domain is essential for GRP94 client maturation
- The GRP94 pre-N domain regulates ATPase activity and dimer closure
- A captured polypeptide fragment in the structure mimics a bound client protein
Figure 1. Architecture of the GRP94 closed dimer

(A) GRP94:AMPPNP contains dimer interfaces at both the N and C domains. Protomers A and B are colored cyan and blue, respectively. (B) The N domain interface is formed by swapping of helices N1 (H1) and strands N1 (S1). The proximal pre-N domains form additional inter-protomer interactions. (C) Rotation about the N-M junction positions Arg448 near the γ-phosphate of AMPPNP and stabilizes closure of the lid (red) over the ATP-binding pocket. (D) The proximal pre-N domain of each protomer is ordered between residues 63–72. The distal pre-N domain is ordered in protomer A between residues 49–60.
Figure 2. GRP94 and TRAP1 pre-N domains have different trajectories

(A) The proximal GRP94 pre-N domain (red) makes few interactions with the opposing N domain (blue) over which it is draped. Insets show that the pre-N crosses strand N6 at an approximate 50° angle and that the 90° turn at Ile66 is guided by Arg237, which makes a hydrogen bond with the backbone carbonyl of Gln65. (B) The TRAP1 pre-N domain (orange) forms an extensive antiparallel β-strand swap with strand N6 and a stabilizing salt-bridge with the opposite N domain (pink). (C) Alignment of isolated N and pre-N domains of GRP94 (blue) and TRAP1 (orange) showing the different pre-N trajectories. (D) Modeling of the TRAP1 pre-N across the N domain of GRP94 reveals that the TRAP1 pre-N would clash with Pro236, Arg237, and Trp333 (yellow).
Figure 3. The pre-N domain is required for GRP94 client maturation and regulates ATPase and dimer closure rates

(A) GRP94 constructs tested for functional complementation in a GRP94\textsuperscript{null} pre-B cell line. Cell surface expression of endogenous GRP94 clients (CD11a, CD49d, and TLR2) was measured by flow cytometry and is expressed as mean fluorescence intensity normalized to wild type (MZG1). (B) ATPase activity of GRP94 was measured as a function of pre-N domain starting residue. Error bars represent the SEM from $\geq$3 independent replicates. (C) The rate of GRP94 or yeast Hsp90 dimer closure following addition of AMPPNP was measured using FRET at 37 or 30 °C for different pre-N domain lengths. Donor and acceptor dyes were introduced at Q453C of the M domain and E131C of the N domain, respectively. Heterodimers were formed by mixing equimolar amounts of donor and acceptor labeled proteins. Fluorescence was recorded at 667nm to monitor changes in acceptor emission. Quantitation of FRET closure rates in panel B. The curve for yeast Hsp90 is shown in Fig. S3D. nd = not determined. (D) Comparison of normalized ATPase and FRET closure rates (37 °C) for selected GRP94 constructs shown in panels B and C. See also Figure S3.
Figure 4. The distal pre-N domain of protomer A mimics an unfolded substrate
(A) 2F_o-F_c electron density for the distal pre-N domain threading through a channel formed by the M and C domains is shown in gray and contoured at 0.95σ (2.0Å carve). (B) Comparison of Cdk4 (yellow) from the Hsp90:Cdc37:Cdk4 complex (PDB: 5FWL) and the distal GRP94 pre-N domain (magenta) based on a superposition of GRP94 and Hsp90 from each structure. (C,D) End-on views of the M:C lumenal channel in GRP94 (C) and Hsp90:Cdk4 (D). (E) Symmetric Gln665 residues in the CBD from each GRP94 protomer (cyan, blue) hydrogen bond with the backbone of the distal pre-N domain (orange). See also Figure S4.
Table 1

Data collection and refinement statistics

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\(^1\) A single crystal was used for data collection