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Characterization of the Grp94/OS-9 chaperone-lectin complex

Paul M. Seidler^{a,b}, Stephen A. Shinsky^c, Feng Hong^d, Zihai Li^d, Michael S. Cosgrove^c, and Daniel T. Gewirth^{a,b,*}

^aDepartment of Structural Biology, University at Buffalo, 700 Ellicott Street, Buffalo, NY 14203, USA

^bHauptman-Woodward Medical Research Institute, 700 Ellicott Street, Buffalo, NY 14203, USA

^cDepartment of Biochemistry and Molecular Biology, SUNY Upstate Medical University, Syracuse, NY 13210, USA

^dDepartment of Microbiology and Immunology; Hollings Cancer Center; Medical University of South Carolina; Charleston, SC 29425, USA

Abstract

Grp94 is a macromolecular chaperone belonging to the hsp90 family and is the most abundant glycoprotein in the endoplasmic reticulum of mammals. In addition to its essential role in protein folding, Grp94 was proposed to participate in the ER associated degradation (ERAD) quality control pathway by interacting with the lectin OS-9, a sensor for terminally misfolded proteins (TMPs). To understand how OS-9 interacts with ER chaperone proteins, we mapped its interaction with Grp94. Glycosylation of the full length Grp94 protein was essential for OS-9 binding, although deletion of the Grp94 N-terminal domain relieved this requirement suggesting that the effect was allosteric rather than direct. Although yeast OS-9 is composed of a well-established N-terminal MRH lectin domain and a C-terminal dimerization domain, we find that the C-terminal domain of OS-9 in higher eukaryotes contains ‘mammalian-specific insets’ that are specifically recognized by the middle and C-terminal domains of Grp94. Additionally, the Grp94 binding domain in OS-9 was found to be intrinsically disordered. The biochemical analysis of the interacting regions provides insight into the manner by which the two associate, and additionally hints at a plausible biological role for the Grp94/OS-9 complex.

Keywords

Chaperone; Hsp90; Grp94; ER associated degradation; Lectin; Intrinsically disordered protein

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*Corresponding Author: Daniel T. Gewirth, Hauptman-Woodward Medical Research Institute, Department of Structural Biology, University at Buffalo, 700 Ellicott Street, Buffalo, NY 14203 USA, Telephone: +1 716-898-8635, Fax: +1 716-898-8660, gewirth@hwi.buffalo.edu.

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Introduction

Grp94 is the endoplasmic reticulum (ER) paralog of cytoplasmic Hsp90 and the most abundant luminal glycoprotein, at times accumulating to concentrations near 100 μ M.^{1; 2; 3} Like all hsp90 chaperones, Grp94 contains an N-terminal (N) ATP binding domain, a middle domain (M), and a C-terminal (C) dimerization domain.⁴ While cytoplasmic Hsp90 employs a retinue of at least 16 co-chaperones in the course of maturing a diverse set of client proteins⁵, to date just one Grp94 co-chaperone, CNPY3, has been identified.⁶ CNPY3 bears no similarity to known Hsp90 co-chaperones and is further distinct in that it is client-specific for Toll-like receptors.

Expression of Grp94 is restricted to higher eukaryotes, and its absence from organisms like yeast suggests that Grp94 is a specialized chaperone with clients consisting of either metazoan-specific proteins or client homologs which differ substantially from their lower eukaryotic counterparts. In addition to chaperoning a select set of surface-expressed or secreted proteins that includes the Toll-like receptors⁷, IgGs⁸, and insulin-like growth factors^{9; 10}, Grp94 also interacts with the lectin OS-9¹¹. OS-9 facilitates the relay of terminally misfolded proteins (TMPs) to a retrotranslocation complex in the ER membrane for proteasomal degradation in the ER associated degradation (ERAD) process,¹¹ thus, at least in principle, providing a linkage between Grp94 and the quality control systems of the ER.

ERAD involves a branched network of convergent pathways that distinguishes nascent proteins from TMPs via the recognition of specific glycan signals.^{12; 13} Nascent proteins entering the ER receive N-linked glycosylations that are subsequently trimmed by mannosidases to reflect the quality of folding. Progressive trimming of mannose from the C-branch of the glycan signals misfolding, cueing recognition by ERAD lectin adaptors like OS-9. The interaction of OS-9 with the TMP is bipartite, with OS-9 binding to the trimmed glycans via its mannose recognition homology (MRH) domain and to the unfolded peptide segments through as-yet unknown regions.^{14; 15; 16} OS-9-bound TMPs are delivered to the HRD1/SEL1 E3 ubiquitin ligase retrotranslocation complex by interaction of SEL1 with the N-terminal portion of OS-9.^{14; 17; 18} Although it is unknown whether Grp94 interacts as a binary complex with OS-9, or in a ternary complex with OS-9 and TMP, evidence suggests that Grp94 dissociates from OS-9 prior to interaction of OS-9 with SEL1.¹¹

While the biological function of the Grp94/OS-9 complex remains a matter of debate, proposed roles include (i) a relay mechanism in which Grp94 hands-off TMPs to OS-9; (ii) a chaperone-client relationship whereby Grp94 assists in folding and/or stabilizing OS-9; and most recently (iii) a proteostatic function in which OS-9 selectively binds to hyper-glycosylated Grp94 (hgGrp94) facilitating the accelerated turnover of hgGrp94 in a proteasome-independent manner¹⁹. A recent *in vivo* mapping analysis carried out in conjunction with the latter study has reported binding of the Grp94 middle domain to a 65 residue peptide sequence in OS-9 (residues 443–507) which also contains the isoform 3/4 splice site.

To date, a notable shortcoming in studies of the ER Hsp90 chaperone is the absence of any quantitative *in vitro* biophysical studies investigating how Grp94, and each of its structural domains, interact with client or co-chaperone proteins. This contrasts with Hsp90, where studies with purified chaperones, client proteins and co-chaperones have characterized the binding parameters of these interactions and, additionally, highlighted the M and C domains as important loci for interaction with clients.^{20; 21; 22; 23} Combined with reciprocal mapping of Hsp90 to specific client motifs, these studies have provided powerful insights into the manner by which Hsp90 associates with its clients.²⁴ To fill this gap in our understanding of how proteins interact with Grp94, and to gain insight into the rationale for the preponderance of ER chaperone binding to cellular OS-9²⁵, we systemically evaluated the contribution of the Grp94 N, M, and C domains to binding OS-9 and reciprocally mapped the interacting portion of OS-9. We report here the results of this biochemical mapping. We find that insertions found within the C-terminal domain of mammalian OS-9 play an important role in the association. Additionally, we find that both the Grp94-M and Grp94-C domains interact with OS-9, and that these binding sites are exposed only in the truncated Grp94-MC, Grp94-M, and Grp94-C constructs, or in the post-translationally modified full length chaperone. Together with the previously discovered interactions involving OS-9 and a repertoire of other ER proteins, our Grp94/OS-9 mapping study provides insight into the mode of interaction and additionally hints at a potential alternative biological role for the complex.

Results

Grp94 binds to the non-lectin portion of OS-9

OS-9 is composed of two domains: a mannose recognition homology (MRH) lectin domain located at the N-terminus and a C-terminal domain that in yeast serves a dimerization function^{17; 18} (see Figure 1 for constructs used in these studies). To determine which OS-9 domain interacts with Grp94, we co-expressed His-tagged Grp94 with full length or truncated OS-9 constructs in *E. coli* and used affinity purification of the His-tagged Grp94 to test for OS-9 association. Because the 73 kDa OS-9 migrates anomalously by SDS-PAGE, with an apparent molecular weight of about 97 kDa²⁶ that overlaps with full length Grp94, co-purification with Grp94 was evaluated by western blotting for OS-9 (Figure 2A). From this analysis, full length OS-9 was detected in the His-Grp94 affinity purified fraction, confirming that complex formation can be detected between the two bacterially co-expressed full length proteins, and consistent with other co-expression analyses that were conducted in cultured mammalian cells.^{11; 12; 25; 27}

To distinguish whether the N-terminal portion of OS-9, which contains the MRH domain (1–229) or the C-terminal portion binds to Grp94, an OS-9 truncation construct (230–666) was co-expressed in bacteria with His-tagged Grp94. To maximize the level of Grp94 expression, a near full length Grp94 composed of residues 73–754 (Grp94-NMC) was substituted for the full length protein.⁴ As seen in Figure 2B, co-expressed OS-9 (230–666) purifies with His-Grp94-NMC, indicating that the MRH lectin domain is not required for binding to Grp94.

OS-9 binds to a site on the Grp94 Middle/C-terminal domain that is exposed only in post-translationally modified full length Grp94

We next used *in vitro* pulldown analysis of purified Grp94 and OS-9 proteins to assess the Grp94 requirements for interaction with OS-9. Because we found that the MRH domain is not required for interaction with Grp94, we used a construct of rat OS-9 comprising residues 267–666 (here termed OS-9 MRH) for these studies. As seen in Figure 2C, purified His-tagged Grp94-NMC that was expressed in Sf21 cells pulls down *E. coli*-expressed, purified untagged OS-9 MRH, recapitulating the bacterial co-expression pulldown experiments described above. Surprisingly, however, the equivalent His-tagged Grp94-NMC construct that was expressed in *E. coli* does not pull down appreciable amounts of purified OS-9

MRH (Figure 2D), suggesting that post-translational modifications on the Sf21-expressed Grp94²⁸ impact OS-9 binding. To determine whether it is the post-translational modifications themselves, or, rather, the conformation of Grp94 influenced by the modifications that is responsible for this discrimination, we tested the ability of a His-tagged Grp94-MC construct that was expressed in bacteria to pull down OS-9 MRH. As seen in Figure 2E, this construct pulls down OS-9 MRH in a manner that is similar to Sf21-expressed Grp94-NMC. The removal of the N-terminal domain appears to be the key requirement for recapitulating the Sf21 Grp94-NMC / OS-9 MRH interaction, since neither the deletion of the charged linker (His-Grp94-NMC 41), the deletion of the C-terminal domain (data not shown), nor the addition of AMPPNP or ADP restores OS-9 binding to the purified bacterially expressed full-length chaperone (Figure 2F and G).

Hsp90 chaperones are known to undergo conformational changes upon ATP binding and hydrolysis that are important for regulating co-chaperone interactions and promoting client maturation.^{29; 30; 31; 32} We thus also tested whether nucleotide binding influenced Grp94/OS-9 complex formation. Using purified His-tagged Sf21-expressed Grp94 and purified untagged OS-9 MRH, we carried out affinity pull-downs in the presence or absence of 1 mM ADP or the ATP mimetic, AMPPNP. The Grp94 protein concentration was set at 30 μ M in order to ensure saturation of the Grp94-nucleotide complex, which as a KD of approximately 4 μ M for both ADP and ATP.³³ As seen in Figure 2H and quantitated in Figure 2I, apo-, ADP-bound, and AMPPNP-bound His-tagged Grp94 all pulled down similar amounts of OS-9 MRH, indicating that the chaperone forms a stable complex with OS-9 that is independent of nucleotide occupancy. This is consistent with previous observations that ATP hydrolysis-deficient mutants of Grp94 maintain binding to OS-9 *in vivo*.³⁴

Both the Grp94 Middle and C-terminal domains bind to OS-9

Pulldowns, co-immunoprecipitations, and *in vivo* association studies provide limited insight into the biophysical parameters of macromolecular interactions. In order to further probe the association of Grp94 and OS-9, we carried out isothermal titration calorimetry (ITC), which provides a label- and matrix-free measure of the affinity of interacting molecules. Pulldown data and the absence of a nucleotide dependence on Grp94's interaction with OS-9 shown above suggested that the OS-9 binding site was in the Middle or C-terminal domains of Grp94. To test this, we determined the binding affinities of the Grp94-MC, Grp94-N, Grp94-M, and Grp94-C domains for OS-9 MRH. Because Grp94 constructs containing the

C-terminal domain are dimeric, and purified OS-9 constructs are predominantly hexameric as suggested by AUC (Supplementary Figure 1), the concentrations of the oligomers, where appropriate, were used in the calculation of dissociation constants. ITC has been used previously to evaluate multivalent ligand-protein complexes.^{35; 36; 37; 38}

As seen in Figure 3 and Supplemental Figure 3, titration of Grp94 domains into OS-9

MRH results in a series of exothermic excursions followed by an endothermic plateau. Since OS-9 binding to Grp94 proceeds with slow kinetics at room temperature (Supplementary Figure 2), ITC experiments were performed at 30 °C in order to overcome the slow rate of association. Subtracting the endothermic heat arising from dilution of the concentrated Grp94 domain titrant produced isotherms that could be fit to a single site binding model. From these studies, which are summarized in Table 1, we find that Grp94-MC binds to OS-9 MRH with an apparent dissociation constant (K_D) of 8.4 μ M. Grp94-M and Grp94-C also bind OS-9 MRH, with K_D 's of 25 and 24 μ M, respectively. The weaker binding of the individual M and C domains to OS-9 MRH, compared to the longer Grp94-MC construct, suggests that the OS-9 binding site is distributed between these two domains. As a control, titration of Grp94-N into OS-9 MRH produced only small endothermic heats of dilution that could not be fit to any binding model (Figure 3B), indicating that the binding of OS-9 is specific to the M and C domains.

To confirm that a *bona fide* Grp94/OS-9 MRH complex was formed during the ITC titration, each completed titration was recovered from the ITC sample cell without additional manipulation and subjected to gel filtration chromatography. As seen in Figure 3C and Supplemental Figure 3C and D, SDS-PAGE of the chromatogram fractions shows that, in addition to the excess free Grp94-MC, Grp94-M, or Grp94-C added in the course of the titration, each Grp94 domain elutes in a higher molecular weight peak that corresponds to a complex with OS-9 MRH (Figure 3D). Gel filtration of the negative control titration of Grp94-N into OS-9 MRH showed only trace amounts of Grp94-N co-eluting with OS-9 MRH (Figure 3E), confirming the lack of binding for this domain as measured by ITC.

Grp94 binds to the C-terminal domain of mammalian OS-9 and to a mammalian-specific OS-9 CTE

To gain further insight into which regions of OS-9 interact with Grp94, the sequences of rat and human OS-9 were aligned with that of yeast OS-9 (yOS9) (Supplementary Figure 4). These alignments revealed that while the MRH domain is conserved across species (27% identity between human and yeast), the C-terminal domains of OS-9 exhibit considerable species variation. In particular, mammalian OS-9 contains 5 insertion segments interspersed throughout the dimerization domain in addition to a large C-terminal extension (CTE) of about 60 amino acids that is not found in yeast (Figure 1A). Even excluding these insertions, the C-terminal domains of yeast and human OS-9 exhibit only 13% identity. By contrast, human and rat OS-9 are very similar to each other, sharing 95% identity in the MRH domains, 73% identity in their C-terminal domains, and 90% identity in the mammalian-specific C-terminal extension. Since yeast do not contain Grp94, we hypothesized that the evolutionarily divergent C-terminal domain found in mammalian OS-9 might contain sites that evolved to bind Grp94.

In order to test the hypothesis that the mammalian OS-9 C-terminal domain serves as a specific Grp94 binding site, we compared the association of Grp94 with rat OS-9 and yOS-9. As seen in Figure 4A, while His-Grp94-MC pulled down rat OS-9 MRH, yOS-9 MRH showed no interaction with Grp94. This lack of interaction between yOS9 and Grp94 highlights the specificity of the Grp94 complex formed with mammalian OS-9.

To evaluate the contribution of mammalian-specific OS-9 inserts to Grp94 binding, we made a series of OS-9 MRH C-terminal truncation constructs (Figure 1A) and used affinity pulldowns to test their ability to bind to Grp94. As seen in Figure 4B and C, removal of amino acids 589–666, which includes the CTE, the largest and most conserved mammalian-specific insert, diminished OS-9 binding to Grp94 by approximately 75%. Further truncation of OS-9 MRH to the dimerization domain (DD, residues 267–486) completely abolished binding to Grp94. This suggests that the major site of Grp94 interaction resides in the OS-9 CTE and a short stretch of preceding residues.

To test whether Grp94 bound directly to the isolated OS-9 CTE, we titrated OS-9 CTE into the bi-domain Grp94-MC and monitored binding by ITC. As shown in Figure 4D and summarized in Table 1, the ITC titration reveals that OS-9 CTE binds to the Grp94-MC with an apparent K_D of 22 μ M. A similar analysis using the mono-domain Grp94-M, and Grp94-C constructs revealed K_D 's of 14, and 15 μ M, respectively (Figure 5A and B). A control titration of OS-9 CTE into Grp94-N produced only endothermic heats of dilution that could not be fit to any binding model (Figure 5E), showing that the binding of OS-9 CTE to Grp94-MC, Grp94-M, and Grp94-C reflects the specific recognition of these domains. Titrations recovered from the ITC sample cell were analyzed by gel filtration, and SDS-PAGE and confirmed the interactions observed by ITC (Figure 5 C, D, and F and Supplementary Figure 5).

OS-9 MRH bound Grp94-MC with higher affinity than Grp94-M and Grp94-C alone, indicating that the OS-9 MRH binding site is distributed between the Grp94-M and -C domains. The lack of an analogous order of affinity between OS-9 CTE and the Grp94-MC, Grp94-M, and Grp94-C constructs, however, was surprising. The modest reduction in the observed affinity of OS-9 CTE for Grp94-MC suggested that the Grp94-M and Grp94-C domains may be competing for the same binding site on the shorter OS-9 CTE. To test this hypothesis, a competition ITC was performed where Grp94-C was titrated into OS-9 CTE that had been pre-incubated with Grp94-M. Indeed, as seen in Supplementary Figure 6, pre-bound Grp94-M effectively blocked Grp94-C binding to OS-9 CTE, indicating that the two domains share an overlapping binding site on the OS-9 CTE.

In summary, the data in Table 1 shows that although both OS-9 MRH and OS-9 CTE bound to Grp94-M and Grp94-C with similar affinities, only OS-9 MRH bound to the Grp94-MC construct with a higher affinity compared to its constituent domains. This suggests that Grp94-MC interacts with multiple discrete epitopes present within the longer OS-9 MRH construct, and that this additional epitope is absent from the OS-9 CTE. A recent report mapped a Grp94 binding site to residues 443–507 of the OS-9 C-terminal domain,¹⁹ and the ITC data presented here are consistent with this being the second binding site detected in the OS-9 MRH construct. Additionally, because the individual Grp94-M

and Grp94-C domains bound each of the OS-9 constructs with similar affinities, this suggests that both domains of the chaperone are capable of recognizing similar features of a partner protein.

Grp94 binds the OS-9 C-terminal domain *in vivo*

In order to evaluate *in vivo* the Grp94 binding sites that we identified *in vitro*, we tested the ability of S-tagged OS-9 deletion constructs to pull down endogenous Grp94 in transiently transfected HEK293 cells. As seen in Figure 6, Grp94 co-purifies with full length S-tagged OS-9 (31–666), S-tagged OS-9 MRH (267–666), and S-tagged OS-9 CTE (31–588). It did not co-purify with the OS-9 MRH domain (31–229) or the more extensive C-terminal deletion construct, OS-9-486 (31–486). These results, which are in agreement with previous *in vivo* pulldown studies¹⁹, confirm the OS-9 C-terminal domain as the site of Grp94 binding. Interestingly, however, while deletion of the OS-9 CTE (residues 589–666) significantly diminished Grp94 binding *in vitro*, transfection of the same OS-9 deletion construct in HEK293 cells had no effect on Grp94 binding *in vivo*. Although the data does not allow us to fully reconcile these differences between the *in vivo* and *in vitro* binding studies, it is possible that deletion of the OS-9 CTE may result in the increased *in vivo* exposure of the previously identified¹⁹ Grp94 binding site comprising residues 443–507 of OS-9.

The C-terminal domain of mammalian OS-9 is intrinsically disordered

Computational predictions made using the program PONDR-FIT³⁹ indicated that the C-terminal domain of mammalian OS-9, to which Grp94 binds, is likely to be disordered (Supplementary Figure 6). This is in contrast to yOS9, which by a similar analysis is predicted to be well folded. To evaluate the structural properties of the mammalian OS-9 C-terminal domain, a circular dichroism (CD) spectrum of OS-9 MRH was collected. As seen in Figure 7A, the most prevalent spectral features are strong minima at 204 and 222 nm. While the band at 222 nm derives from α -helical structure, the band at 204 nm indicates the presence of intrinsically disordered regions and arises from the combination of two minima, one at 200 nm arising from random coil and the other at 208 nm arising from α -helical content.^{40; 41}

Since the CD spectrum of the OS-9 MRH indicated a mixture of disordered peptide and α -helical structure, we wanted to assess its overall foldedness. While well-folded proteins classically denature with a cooperative transition, proteins containing a high proportion of unstructured polypeptide exhibit non-cooperative transitions.⁴⁰ A thermal denaturation scan for OS-9 MRH showed a linear decrease in ellipticity at 222 nm with increasing temperature (Figure 7B), ultimately reaching a plateau at 85 °C, suggesting that the C-terminal domain of OS-9 is intrinsically disordered. A similar CD spectral analysis of the isolated OS-9 CTE, a major *in vitro* Grp94 binding site, showed minima at 208 and 222 nm suggesting a predominantly helically structured peptide (Figure 7C). Surprisingly however the OS-9 CTE also denatured with a linear transition, suggesting that the CTE, despite having helical properties, possesses poor conformational and/or thermodynamic stability (Figure 7D).

To further probe the global structural state of OS-9, we carried out limited tryptic digests on OS-9 MRH and OS-9 CTE. Control digests were carried out on Grp94-M (residues 337–629), which is well folded, and α -casein, which is intrinsically disordered.⁴² As seen in Figure 7E, OS-9 MRH exhibits little resistance to limited tryptic digest. Substantial cutting was observed at the intermediate trypsin concentration and complete digestion of the full length polypeptide was seen at the highest trypsin concentration. The cleavage pattern also reveals that OS-9 MRH is digested into numerous lower molecular weight species indicating that the C-terminal domain of mammalian OS-9 is predominantly in the form of an extended polypeptide. The tryptic digest pattern of OS-9 MRH closely matched that of α -casein, which is a known intrinsically disordered protein. The OS-9 CTE likewise showed a high degree of protease sensitivity, virtually mirroring the cleavage pattern and susceptibility of α -casein. By contrast, Grp94-M, which is predicted to have low disorder (Supplementary Figure 6C), exhibited stronger resistance to trypsin, showing substantial cleavage into a single lower molecular weight species only at the highest protease concentration. Collectively, disorder predictions, CD spectra, and limited proteolysis experiments establish that the C-terminal domain of OS-9, to which Grp94 binds, is largely unstructured.

Discussion

In this report, we have carried out the first biophysical analysis of the interaction between the ER hsp90 chaperone, Grp94, and a partner protein. We have shown that Grp94 uses its middle and C-terminal domains to bind a mammalian-specific C-terminal domain of OS-9. The interaction of Grp94 with OS-9 is complex, involving both a recently identified OS-9 epitope in the C-terminal portion of the protein¹⁹, as well as an additional site in the C-terminal extension (CTE). The OS-9 binding site on Grp94 is exposed only in the post-translationally modified intact chaperone or in the unmodified chaperone that lacks the N-terminal domain, suggesting that these modifications promote a unique conformation of Grp94. The interaction between Grp94 and OS-9 is restricted to mammalian OS-9, as the evolutionarily divergent yeast OS-9 shows no interaction with Grp94. We have further shown that the region of OS-9 recognized by Grp94 exhibits biochemical and biophysical characteristics of an intrinsically disordered protein, suggesting that the interaction with Grp94 may reflect the need to stabilize OS-9 *in vivo*.

Unlike the less well studied hsp90 paralog Grp94, numerous complexes between cytoplasmic Hsp90 and its co-chaperones, along with a growing number of client proteins, have been analyzed biochemically. Based on these studies, Hsp90 partner proteins can be grouped into two broad categories: those exhibiting high-to-moderate affinities (ranging from about 0.1 to 10 μ M) and those exhibiting moderate-to-low affinities (ranging from about 1 to 100 μ M). Hsp90/co-chaperone complexes fall predominantly into the high affinity class, and are characterized by interactions between discrete domains and sequence motifs of the interacting partners.^{43; 44; 45; 46; 47}

By contrast, Hsp90 client complexes exhibit significant biochemical parallels to the Grp94/OS-9 complex studied here. These complexes are characterized by moderate-to-low affinity interactions and by the use of multiple protein-chaperone contacts that span two or more

domains of the chaperone.^{20; 22; 23; 24; 48; 49} Thus, for example, in the interaction between Hsp90 and the client protein p53, each Hsp90 domain forms measurable interactions with p53. The intact chaperone, and presumably all 3 domain interactions, however, is required to stabilize p53 *in vitro*^{21; 22; 23}.

The association of HtpG with 131, a metastable surrogate client,²⁰ and Hsp90 with cytosolic kinases provide evidence that Hsp90 binding can be driven by a client protein's thermodynamic instability, paralleling the interaction of Grp94 with OS-9. As with Grp94/OS-9, the 131 binding site on HtpG spans the middle and C-terminal domains and the N-terminal domain is dispensable for the association.^{48; 50} HtpG binding was mapped to a marginally structured region in the globally unfolded 131 protein. For kinases, an extensive analysis of kinase-Hsp90 complexes showed that Hsp90 binds preferentially to kinases with poor stability, and that stabilization of these kinases reduces the level of Hsp90 association²⁴. Additionally, Hsp90 binding determinants for kinase clients are widely distributed throughout the protein sequence, combinatorially contributing to the complexes' overall affinity. Both of these interactions parallel the observations made here that Grp94 binds a marginally structured CTE segment of OS-9 which resides in a predominantly disordered C-terminal domain. Furthermore, the ITC binding analysis shows that while the CTE of OS-9 is important for association with Grp94, the affinity of Grp94 for the isolated CTE is less than that measured for the intact OS-9 C-terminal domain, suggesting that multiple OS-9 sites contribute to the overall interaction.

Although several *in vivo* studies have previously reported the interaction of Grp94 with OS-9,^{11; 12; 25; 27} it remained to be clarified whether the Grp94/OS-9 complex formed a stable binary complex or formed only in cooperation with TMPs as a ternary complex.^{11; 27; 28} While the present study clearly demonstrates that a direct stable interaction is formed between purified Grp94 and OS-9, our results do not rule out the possibility that formation of a ternary complex somehow further enhances the association.

A recent *in vivo* analysis showing that TMPs bind poorly to Grp94 argues in favor of a binary Grp94/OS-9 complex.¹⁹ The same study mapped binding to the middle domain of Grp94 and to a region near the C-terminus of OS-9 spanning residues 443–507 (termed 94BR). In agreement with that report, we find that the Grp94 middle domain binds OS-9 and that a series of residues which overlap with the identified 94BR contribute to the association. Additionally however, our *in vitro* analysis reveals interactions involving a second binding site in OS-9 that is localized to a 78 residue stretch (termed the CTE) that is proximal to the 94BR. In addition to identifying a larger surface on OS-9 that interacts with Grp94, we also observed a larger OS-9 binding region on Grp94 that encompasses both the middle and C-terminal domains. Given the relatively low affinity we have measured for these interactions, it is possible that the binding surfaces identified *in vivo* correspond to a partial binding site which overlaps with the site we have mapped *in vitro*.

While our biophysical experiments demonstrate that the OS-9 CTE is a major Grp94 binding site *in vitro*, co-IP experiments revealed that deletion of the OS-9 CTE does not disrupt Grp94 binding in HEK293 cells. This may be due to an increased exposure of the remaining Grp94 binding site, 94BR, after deletion of the OS-9 CTE, possibly with the assistance of

other chaperones in these cells. Because the deletion of the CTE significantly reduced Grp94 binding to OS-9 *in vitro*, it is possible that 94BR is partially obscured in the hexameric OS-9 oligomer used for our *in vitro* study.

In agreement with the *in vivo* mapping study published previously that reported a selective interaction of OS-9 with hyper glycosylated Grp94¹⁹, we observed a requirement for post-translationally modified full-length Grp94 in our *in vitro* affinity pull-down experiments. The fact that similar OS-9 binding could be observed with the un-modified Grp94-MC construct, however, suggests that the role of the Grp94 modifications is not to present a carbohydrate-based recognition motif to OS-9, but, rather, to potentiate a Grp94 conformation that exposes the OS-9 binding sites on the Grp94-M and Grp94-C domains. By this reasoning, hyper glycosylation of Grp94 may further shift the conformation of the chaperone to the OS-9 binding state, thereby accounting for the observed increase in the interaction with OS-9. The conformational consequences of modifications to Grp94 or the other Hsp90 chaperones that might lead to the exposure of otherwise obscured client binding sites are poorly understood at this time.

On the basis of the apparent glycan-dependent interaction of OS-9 with Grp94, it was proposed that hyper glycosylated Grp94 becomes preferentially bound to OS-9 thereby facilitating its selective degradation by a non-proteasomal pathway. Although feasible, our *in vitro* analysis shows that non-glycosylated Grp94 binds to a conformationally unstable segment of OS-9, and leads us to propose an alternative model in which Grp94 functions to sequester potentially toxic peptides by binding to the OS-9 C-terminal domain, as we outline below.

The notion that OS-9 may be unstable and requires chaperone stabilization *in vivo* is supported by mass spectrometry studies showing that OS-9 is predominantly found in complex with the ER chaperones Grp94 and BiP, while only a fraction interacts with TMPs and components of the retrotranslocation machinery.^{11; 25} This disproportionate distribution suggests that the intrinsically unstable nature of OS-9, which we have demonstrated through our biophysical study, may also translate to the cellular environment. Accordingly, we propose that both Grp94 and BiP are capable of binding and sequestering OS-9, and that this activity represents a general chaperoning phenomenon. Grp94 may be particularly well suited for OS-9 binding, however, owing to its high luminal concentration, which could facilitate the interaction despite the complexes' moderate affinity, while still allowing for facile exchange of chaperone with ERAD substrates. The generality of this chaperone interaction could also explain the apparent lack of a nucleotide-dependent Grp94 binding to OS-9, which is characteristic of canonical hsp90- client and co-chaperone interactions. In support of this, the interaction of Hsp90 with the aggregation-prone protein Tau was also reported to lack nucleotide-dependent binding *in vitro*, demonstrating that nucleotide dependence may not be an absolute requirement for some of the more general housekeeping functions carried out by these chaperones.⁴⁹

In summary, we find that Grp94 binds to a highly conserved segment found exclusively in mammalian OS-9 at the end of an intrinsically disordered C-terminal domain. While yOS9 is primarily dimeric, our studies reveal a strong oligomeric propensity for mammalian OS-9.

Although there is no evidence to suggest that OS-9 oligomers possess a direct biological function, its propensity to oligomerize may rationalize OS-9's apparent affinity for ER chaperones and could highlight a property exploited by mammalian OS-9 to establish peptide-based interactions with TMPs. Future investigations will be needed to clarify the biological role OS-9 oligomerization plays, if any, and the extent to which oligomerization effects Grp94 binding to the two identified sites, 94BR and CTE. Collectively, our experiments show that the OS-9 partner protein bears partial hallmarks of an hsp90 client in that it exhibits a metastable fold and involves multi-site interactions with the middle and C-terminal domains of Grp94 that are characterized by moderate-to-low affinity. However, OS-9 lacks many of the critical hallmarks classically observed for client proteins since Grp94 is not exclusively required for its folding or stability, and OS-9 lacks nucleotide-dependent binding to Grp94. As such, the biological function of the complex still remains largely enigmatic.

Materials and Methods

Reagents

All DNA constructs were confirmed by sequencing. The cDNA for rat OS-9 was purchased from Open Biosystems, OS9 antibody was purchased from Abcam (ab-19853), and goat anti-rabbit alkaline phosphatase conjugate secondary antibody from Santa Cruz (sc-2034).

Protein preparation in *E. coli*

All proteins were expressed in Rosetta™ 2(DE3) *E. coli* cells (Novagen). Rat OS-9 (which shares 83% identity to human OS-9) and its truncation fragments were expressed as N-terminal His-SUMO fusion proteins in a culture grown to mid-log phase at 37 °C followed by overnight induction at 20 °C with 0.5 mM IPTG. Unless otherwise noted, the OS-9 MRH truncation mutant (MRH) comprised amino acids 267–666, the C-terminal truncation mutant (MRH/CTE) comprised amino acids 267–588, and the OS-9 CTE comprised amino acids 589–666. Similarly yeast OS-9 MRH (250–527) was expressed as pET-SUMO fusion. All OS-9 proteins were purified by Ni-NTA affinity chromatography using a standard lysis buffer (50 mM Tris-HCl (pH 8.0), 500 mM NaCl, 20 mM imidazole, and 1 mM β-mercaptoethanol (BME)). The His-SUMO fusion proteins were step-eluted in “imidazole elution buffer” (50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 400 mM imidazole, and 1 mM BME) and peak fractions were pooled and dialyzed against an identical buffer except with 20 mM imidazole. The His-SUMO fusion was cleaved, unless otherwise indicated, by addition of Ulp1 protease for a minimum of 3 hours at room temperature using a protease ratio determined empirically through small scale digest tests. His-SUMO was removed by subtractive Ni-NTA affinity chromatography and, for all OS-9 constructs other than the CTE and yOS-9 MRH, further purification was carried out by FPLC Resource Q ion exchange with an NaCl elution gradient from 250 to 450 mM in 50 mM Tris-HCl (pH 8.0) buffer. For OS-9 CTE, ion exchange chromatography was performed with Sepharose Fast Flow ion exchange resin and eluted using a 250 to 1,000 mM NaCl gradient, and for yOS-9 MRH a gradient from 50–600 mM NaCl. As a last step, OS-9 proteins were passed over a Superdex 200 gel filtration column in 25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM tris(2-carboxyethyl)phosphine (TCEP) for OS-9 CTE or 25 mM HEPES-NaOH

(pH 7.5), 250 mM NaCl, 5% glycerol for all other OS-9 constructs before concentrating by ultrafiltration using a 50 kDa molecular weight cut off spin filter. For all of OS-9 constructs, the flow through was monitored spectrophotometrically at 280 nm and no low molecular weight forms of OS-9 were observed in the flow through. Protein stocks were either used immediately or flash frozen at 5 mg/ml (OS-9 MRH), 15 mg/ml (OS-9 CTE), or 12 mg/ml yOS-9 MRH and stored at -80°C .

Canine Grp94 MC, M, or C proteins were expressed in *E. coli* as N-terminal His-tagged fusions by overnight induction of a culture grown to mid-log at 37°C with 0.5 mM IPTG at 20°C . Unless otherwise noted, Grp94-MC comprised amino acids 337–765, the Grp94-M comprised amino acids 337–594, and Grp94-C comprised amino acids 601–754. His-tagged proteins were purified by Ni-NTA affinity chromatography using a “standard lysis buffer” and eluted in a 50 mM Tris (pH 8.0), 250 mM NaCl, 1 mM BME buffer with a 50 to 400 mM imidazole gradient. For Grp94-MC, combined Ni-NTA fractions were diluted 5-fold in 50 mM Tris (pH 8.0), applied to a Q Sepharose Fast Flow ion exchange column, and eluted in a 50 mM Tris (pH 8.0), 1 mM BME buffer with a 100 to 500 mM NaCl gradient (Grp94-MC). Finally, Grp94-MC was purified by size exclusion chromatography on a Superdex 200 column in a 10 mM Tris (pH 7.6), 250 mM NaCl, 1 mM dithiothreitol buffer before concentrating by ultrafiltration to 30 mg/ml for storage at -80°C . Grp94-M and -C were similarly purified by gel filtration except on a Superdex 75 gel filtration column with a final NaCl concentration of 150 mM. Canine Grp94 69–337 278–327 (Grp94-N) was expressed as GST-fusion and purified as described previously.⁵¹

Co-expression of proteins in *E. coli* was achieved as described above except using the polycistronic expression vector pST39⁵² by cloning the His-tagged Grp94 full length (22–804) or NMC (73–754) into cassette 1 and OS-9 full length (26–666) or MRH truncation (230–666) into cassette 4. His-Grp94 was isolated from lysates by Ni-NTA affinity purification and probed for presence of untagged OS-9 by western blot.

Grp94 expression and purification from insect cells

Canine Grp94 NMC protein comprising amino acids 73–754 was expressed in insect cells using a baculovirus expression system. Briefly, the Grp94 signal sequence (residues 1–23) was fused to N-terminus of Grp94 NMC and a 6× His-tag followed by a KDEL ER retention signal to the C-terminus. Recombinant bacmid was produced in DH10Bac cells, transfected into Sf21 insect cells, and amplified in Sf-900 II SFM supplemented with 1.5% (v/v) fetal bovine serum and antibiotic-antimycotic (100 units/ml penicillin, 100 µg/ml streptomycin, 25 µg/ml amphotericin B). For protein expression, Sf21 insect cells were infected with p3 virus stock at a multiplicity of infection (moi) of about 1.0 when cells reached a density of 2.0×10^6 cells/ml. Cells were grown at 27°C in a spinner flask with air-sparging and lysed using a microfluidizer when viability dropped to 40–60%. Cell debris was cleared by ultracentrifugation at 40,000 rpm in a Ti45 for 45 min, and purified as described above for bacterially expressed Grp94.

Western Blots

Crude lysates from co-expression experiments were separated on a 12.5% SDS polyacrylamide gel and transferred to a nitrocellulose membrane using the PhastGel system (GE Healthcare). Membranes were blocked with 3% gelatin, incubated with a 1/1000 dilution of rabbit polyclonal OS-9 antibody for 1h followed by a goat anti-rabbit alkaline phosphatase conjugated secondary antibody. Blots were developed using an alkaline phosphatase substrate kit (Bio-Rad) according to the manufacturer's instructions.

Affinity pull-downs

Purified Grp94 constructs were diluted to 30 μ M in binding buffer (40 mM sodium phosphate (pH 7.0), 250 mM NaCl, 20 mM imidazole) and, for experiments shown in Figure 2C, supplemented with 1.5 mM $MgCl_2$ and pre-incubated for 30 min with either 1 mM ADP or AMP-PNP, or no nucleotide. An equimolar amount of OS-9 was mixed with Grp94 to a final volume of 200 μ l and incubated at room temperature for at least 16h before addition of 70 μ l Ni-NTA agarose beads. Complexes were incubated with rocking for 30 min at room temperature and subsequently pulled-down by centrifugation at 500 $\times$ g (2,300 rpm) for 30 sec. The supernatant was removed and the resin was washed four times with 1 ml of wash buffer (50 mM Tris-HCl (pH 8.0), 250 mM NaCl, 5% glycerol, and 20 mM imidazole). Finally, resin was washed with 1 bed volume of wash buffer for analysis by SDS-PAGE and bound proteins were eluted with 1 bed volume of "imidazole elution buffer" and samples were resolved by SDS-PAGE. For Grp94 NMC pull-downs shown in Figure 2C which evaluate nucleotide dependence, band intensities of coomassie stained gels were determined for the Grp94 doublet (which arises from differentially glycosylated species⁵³) and OS-9 in the elution lanes by densitometry using ImageJ software⁵⁴. The fraction of OS-9 co-eluting with Grp94, determined from the average of three independent experiments, was then plotted with respect to nucleotide (Figure 2D).

Isothermal titration calorimetry (ITC)

All ITC experiments were carried out using a VP-ITC calorimeter (Microcal) at 30 °C. Titrations involving OS-9 MRH were carried out in an ITC buffer 1 (25 mM HEPES-NaOH (pH 7.5), 250 mM NaCl, 5% glycerol) and titrations with OS-9 CTE were carried out in ITC buffer 2 (25 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM TCEP). All Grp94 and OS-9 proteins were buffer matched by gel filtration chromatography or dialysis and complete buffer exchange was verified by injection of each titrant into buffer. Heats of dilution of the highly concentrated protein titrants were corrected for by subtracting the average value of a saturated baseline at the end of each titration from the binding isotherms. Binding parameters were determined by fitting to a one site model using Origin software. Protein concentrations used for affinity calculations for OS-9 MRH and CTE assumed a hexamer and for Grp94-MC and Grp94-C assumed a dimer. Following ITC injections, samples were recovered from the experimental cell and injected without further manipulation onto a Superdex S200 gel filtration column equilibrated with the ITC buffer. Peak fractions from the gel filtration were analyzed by SDS-PAGE to evaluate complex formation by co-elution of titrant and analyte.

Circular dichroism (CD) spectroscopy

CD spectra were collected for OS-9 MRH (267–666) or CTE (589–666) at a concentration of 50 µg/ml in a 10 mM potassium phosphate (7.4) buffer containing 100 mM ammonium sulfate on a JASCO J-715 spectrophotometer. Spectra were collected over the range 190–260 nm at 20 °C in a 1 cm cuvette. Temperature interval measurements were similarly acquired every 1 °C over a temperature range of 10 – 100 °C using in a capped 1 cm cuvette.

Limited Proteolysis

Limited digests were performed using OS-9 CTE (11 trypsin sites), OS-9 MRH (50 trypsin sites), Grp94 337–629 (54 trypsin sites), or bovine α-casein (30 trypsin sites) obtained from MP Biomedicals at a protein concentration of 2 mg/ml by addition of trypsin to a final concentration of 0.5 µg/ml, 5 µg/ml, or 10 µg/ml. Reactions were incubated for 1 hour at 4 °C, quenched by the addition of SDS-PAGE buffer, and electrophoresed on 8–25% SDS polyacrylamide gels.

Analytical Ultracentrifugation

Various concentrations of protein were loaded into 12 mm two-sector charcoal-filled Epon centerpieces with quartz windows. Sedimentation velocity experiments were carried out at 20°C or 37°C using a Beckman Coulter ProteomLab XL-A analytical ultracentrifuge equipped with absorbance optics and a 4-hole An-60 Ti rotor at 60,000 RPM ($262,000 \times g$) that was pre-equilibrated to the experimental temperature for two hours. The samples were scanned at 0-min time intervals for 300 scans and analyzed by the continuous distribution $c(s)$ method in the program SEDFIT⁵³. The program SEDNTERP was used to correct the experimental s -value (s^*) to the standard conditions at 20°C in water ($s_{20,w}$) and to calculate the frictional ratio of each sedimenting species⁵⁴.

HEK293 transfection and co-IP

All DNA constructs were made by cloning the coding sequence from rat OS-9 (residues 31–666) into a pcDNA3 vector which was modified to contain the bovine preprolactin ER signal sequence followed by an S-tag. HEK293T cells were transiently transfected with 20 µg of S-tagged wild type or mutant OS-9 plasmids for 48 hours, and then lysed in RIPA buffer (1% NP40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 150 mM NaCl, 2 mM EDTA, 0.01 M sodium phosphate pH 7.2 supplemented with protease inhibitor cocktail). Protein concentration of the postnuclear lysate was quantified by Bradford assay (BioRad) and 1.5 mg of lysates were incubated with 3 µg of anti-S tag antibody coupled to S tag beads at 4 overnight (Novagen). The antibody–bead complexes were then extensively washed with RIPA buffer. The bound proteins were eluted by heating the beads at 95 °C in reducing SDS-loading buffer. Co-immunoprecipitated proteins were resolved in 8% SDS PAGE gel, followed by immunoblotting. The whole cell lysates (WCL) were resolved in the same gel as inputs.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Biochemical parameters for Grp94-protein interactions with cellular proteins are unknown
- OS-9 binding to Grp94 requires glycosylation of the full length chaperone protein
- The middle and C-terminal domains of Grp94 were found to bind OS-9
- Grp94 binds to unique inserts found near the C-terminus of mammalian OS-9
- The C-terminal domain of OS-9 is intrinsically disordered

Figure 1. OS-9 and Grp94 protein constructs used in these studies

(a) OS-9 constructs from rat or yeast, as indicated. Grey hashed boxes in the rat OS-9 sequence are insertions found only in the mammalian protein. Corresponding regions found in the yeast protein are indicated by a dashed line. **(b)** Grp94 constructs used in this study. For the constructs indicated, the “charged linker” (CL) was replaced with a four glycine linker (4×Gly). ss = signal sequence, MRH = mannose recognition homology domain, L = proline-rich linker, CTE = mammalian C-terminal extension, N = N-terminal domain, M = middle domain, and C = C-terminal domain.

Figure 2. Grp94 binds to the C-terminal domain of OS-9

(a) SDS gel of bacterially co-expressed OS-9 and His-Grp94 following Ni-NTA affinity purification. The bands for OS-9 and His-Grp94 overlap. Left panel, coomassie stained, right panel, OS-9 Western blot. S = supernatant, E = elute. (b) SDS gel of bacterially co-expressed OS-9 MRH (230–666) and His-Grp94-NMC (73–754). Left panel, coomassie stained, right panel, OS-9 Western blot. (c) Ni-NTA affinity pull-down with OS-9 MRH (267–666) and His-Grp94 NMC expressed in Sf21 insect cells (with post-translational modifications, PTMs). (d) as in C except using His-Grp94 expressed in E. coli (without

PTMs). **(e)** as in C except using His-Grp94-MC expressed in *E. coli*. **(f)** as in (c) except using His-Grp94-NMC + 1 mM AMP-PNP. **(g)** as in (c) except using His-Grp94-NMC 41 expressed in *E. coli*. **(h)** Ni-NTA affinity pull-downs using purified OS-9 MRH and His-tagged GRP94-NMC expressed in Sf21 insect cells. Pulldowns were carried out in the absence (APO) or presence of 1 mM ADP or AMP-PNP. Grp94 doublet band reflects differential phosphorylation and glycosylation states characteristic of Grp94 overexpression in insect cells. **(i)** Band intensities from Grp94/OS-9 MRH pull-downs (as shown in C) were quantified by densitometry and the fraction of OS-9 co-eluting with Grp94, determined from the average of three independent experiments, was plotted as a function of ligand.

Figure 3. Grp94-MC binds to OS-9 MRH

(a) ITC titration of 352 μM Grp94-MC dimer titrated into 48 μM OS-9 MRH hexamer.

The endothermic heat of dilution was determined from the saturated baseline and subtracted from the exothermic binding reaction. **(b)** ITC of 341 μM Grp94-N titrated into 48 μM OS-9 MRH hexamer.

(c) Top, S200 gel filtration of purified Grp94-MC titrated into buffer and recovered from the ITC cell; Bottom, S200 gel filtration of the mixture of Grp94-MC and OS-9 MRH recovered from the ITC titration in (a).

(d) Top, Coomassie stained SDS gel of

the peak fractions in panel (c), top. *Bottom*, Coomassie stained SDS gel of the peak fractions in panel (c), bottom. **(e)** As in (d) above except for Grp94-N and OS-9 MRH.

Figure 4. GRP94 MC binds a C-terminal extension of mammalian OS-9

(a) Coomassie stained SDS gel of Ni-NTA affinity pull-down of His-Grp94-MC with OS-9

MRH from rat (267–666) or yeast (266–527). S = supernatant, W = wash, E = elute. (b)

Coomassie stained SDS gel (8–25% top and middle, 12.5% bottom) of Ni-NTA affinity

pull-downs using His-GRP94-MC and rat OS-9 truncation constructs. (c) Band intensities

from Grp94-OS-9 pull-downs (shown in (b)) were quantified by densitometry and the

fraction of OS-9 co-eluting with His-Grp94 was plotted for each OS-9 construct shown in

(a). (d) ITC titration of 1.6 mM OS-9 CTE hexamer into 83 μM GRP94-MC dimer. Heat of

dilution was determined from the saturated baseline and the binding isotherm was fit to a

one-site model.

Figure 5. GRP94 Middle and C-terminal domains bind OS-9 with similar affinities

(a) ITC titration of 1.59 mM OS-9 CTE hexamer into 81 μ M Grp94-M. (b) Coomassie stained SDS gel of the recovered ITC titration in (a) following gel filtration on S200. The top panel is purified Grp94-M, and the bottom panel is the post-ITC mixture of Grp94-M and OS-9 CTE. (c) ITC titration of 1.57 mM OS-9 CTE hexamer into 77 μ M Grp94-C dimer. (d) Coomassie stained SDS gel of the ITC titration in (c) following gel filtration on an S200 column. (e) ITC titration of 1.4 mM OS-9 CTE hexamer into 79 μ M Grp94-N. (f) Coomassie stained SDS gel of the ITC titration in (e) following S200 gel filtration.

Figure 6. Grp94 binds the CTD of OS-9 *in vivo*

EK293 cells were transfected with S-tagged rat OS-9 constructs encoding the MRH domain (residues 31–229), the MRH domain (residues 267–666), the full length (residues 31–666), or C-terminal truncation constructs 486 (residues 31–486) or 589 (residues 31–589). Cell lysates were immunoprecipitated using S-protein agarose beads and western blotted as indicated.

Figure 7. The OS-9 C-terminal domain is intrinsically unstable

(a) Circular dichroism spectra of OS-9 MRH (267–666) collected at 50 μg/ml in phosphate buffer. (b) Change in ellipticity at 222 nm as a function of temperature. (c and d) As in (a and b) except for OS-9 CTE (589–666). (e) Limited trypsin digest of 2mg/ml OS-9 MRH, OS-9 CTE, α-casein, or GRP94-M (337–629) at trypsin concentrations of 0: 0 μg/ml, 1: 0.5 μg/ml, 2: 5 μg/ml, or 3: 10 μg/ml. Asterisk marks α-casein aggregate.

Table 1

Thermodynamic parameters for the interaction of GRP94 and the OS-9 hexamer determined by ITC at 303 K. Apparent dissociation constants (K_D), expressed in μM , enthalpy (ΔH) and entropy (ΔS) expressed in kcal/mol, and stoichiometries (N) expressed as a ratio of Grp94 monomer (N , M) or dimer (MC , C) to the OS-9 hexamer. Abbreviations: binding not detected (nd).

	OS-9 MRH			OS-9 CTE			
	K_D	N	ΔH	ΔS	K_D	N	ΔH
Grp N	nd	nd	nd	nd	nd	nd	nd
Grp M	25.4 ± 2.6	2.8	-3.3 ± 0.1	3.1	14.3 ± 2.1	3.4	-9.1 ± 1.0
Grp C	24.6 ± 1.7	1.0	-14.0 ± 0.7	-7.6	15.0 ± 1.1	2.9	-33.0 ± 1.7
Grp MC	8.4 ± 2.3	0.15	-7.2 ± 2.3	-0.1	22.4 ± 2.0	2.7	-11.6 ± 1.0