Radicicol-sensitive Peptide Binding to the N-terminal Portion of GRP94*

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GRP94 is a molecular chaperone that carries immunologically relevant peptides from cell to cell, transferring them to major histocompatibility proteins for presentation to T cells. Here we examine the binding of several peptides to recombinant GRP94 and study the regulation and site of peptide binding. We show that GRP94 contains a peptide-binding site in its N-terminal 355 amino acids. A number of peptides bind to this site with low on- and off-rates and with specificity that is distinct from that of another endoplasmic reticulum chaperone, BiP/GRP78. Binding to the N-terminal fragment is sufficient to account for the peptide-binding activity of the entire molecule. Peptide binding is inhibited by radicicol, a known inhibitor of the chaperone activities of HSP90-family proteins. However, the peptide-binding site is distinct from the radicicol-binding pocket, because both can bind to the N-terminal fragment simultaneously. Furthermore, peptide binding does not cause the same conformational change as does binding of radicicol. When the latter binds to the N-terminal domain, it induces a conformational change in the downstream, acidic domain of GRP94, as measured by altered gel mobility and loss of an antibody epitope. These results relate the peptide-binding activity of GRP94 to its other function as a chaperone.

GRP94 has long been inferred to be a peptide-binding protein because of its ability to augment presentation of peptides to T cells (1). Direct evidence for peptide binding was obtained in recent years by identification of peptides in acid eluates of purified GRP94 (2, 3) and by direct binding of several peptides to purified GRP94 (4, 5). A number of exciting studies, both in vitro and in vivo, showed that GRP94 introduces its bound peptides into professional antigen presenting cells, by endocytosis via the CD91 receptor (6–8) or via a CD91-independent pathway (9), thereby dramatically increasing peptide recognition by T cells (6–8). These studies point to immunization with GRP94 as a potentially effective tool to boost immune responses that are otherwise very weak.

To better utilize GRP94 as a vaccine, the properties of peptide binding need to be characterized. An unusual property of GRP94 is that its peptide binding is stimulated by treatments that are expected to destabilize the protein, such as heat shock (7) or guanidinium hydrochloride (10). The peptide binding activity of GRP94 is also stimulated by bis-ANS,1 which binds to the same N-terminal domain as adenosine nucleotides, the ansamycin antibiotic geldanamycin, and another fungal metabolite, radicicol (11). Such stimulation of peptide binding is attributable to conformational changes in the chaperone, reflected in oligomerization and increased binding of hydrophobic dyes, but which are as yet only partly mapped and defined.

The sequence analysis of GRP94 and its comparison with the known structure of HSP90 predicts GRP94 to be a multidomain protein. The putative N-terminal domain (amino acids 1–263) is highly homologous to the proteolytic fragment of HSP90 encompassing residues 9–232, which was shown to be the radicicol/geldanamycin/nucleotide-binding domain (12, 13). It is followed by a sequence (amino acids 264–344) with many runs of acidic residues, which contains the recognition site for the commonly used monoclonal anti-GRP94 antibody (14). Both the putative N-terminal domain and the acidic domain are required for binding of nucleotides and inhibitors to GRP94 (15). A peptide-binding site was defined within the C-terminal domain of GRP94 by cross-linking pyrene-modified VSV8 (16, 17), an octapeptide known to be presented via GRP94 to T cells (3). This site, centered around amino acids 624–630, was modeled as a shallow surface groove formed by α helices, resembling the peptide-binding site of a major histocompatibility locus protein (16). Site-directed mutations showed that VSV8 binding to this site is dependent on aromatic side chains for binding affinity (17). Fluorescence polarization and energy transfer experiments suggested that peptide was bound to higher order GRP94 assemblies (18, 19), as was also proposed for the homologous cytosolic chaperone HSP90 (20). All of these sites were shown to be needed for binding of substrates (21–24), and two separate peptide-binding and/or chaperone sites with different specificities were proposed (22, 23). In the former study, only the C-terminal site bound peptides, whereas the chaperone site in the N-terminal fragment was sensitive to geldanamycin. In the latter study, the N-terminal site was capable of binding peptides with preference for 10 residues or longer, in ATP-dependent fashion (23). It is possible, therefore, that GRP94 also possesses two peptide/substrate binding sites. To characterize peptide binding by GRP94 we undertook a different approach,

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1 The abbreviation used is: ANS, 1,1′-bis(4-anilino-5-naphthalenesulfonic acid).
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defining the peptide binding capability in solution of truncated and in-frame-deleted versions of the full-length GRP94. In the work described here we find a different peptide-binding site, capable of binding a number of peptides, within the first 355 amino acids of GRP94. We show that peptide binding to this site is inhibited by known ligands of GRP94 and that the inhibition is due to transmission of a conformational change along the chaperone.

EXPERIMENTAL PROCEDURES

Purified Proteins—Full-length murine GRP94 was expressed and purified as recombinant protein in insect cells. The expression construct for GRP94 contained a His6 tag at its N terminus, followed by a TEV protease cleavage site. It lacked the signal sequence of the protein and harbored one amino acid substitution, T198I, ablating the one glycosylation site, which is usually used in mammalian GRP94. As shown previously non-glycosylated GRP94 binds to its cellular substrates as efficiently as the glycosylated protein (25). This expression construct was made as follows: a GRP94 cDNA clone (pGEM99.2; Ref. 26); a generous gift from Dr. M. Green, St. Louis University, MO was used as a PCR template. The substitution T198I was introduced into this clone and then the cDNA insert (without the signal sequence) was PCR-cloned into pHJHTb baculovirus expression vector (Invitrogen). The construct was verified by sequencing. In numbering amino acids of GRP94 we designate as +1 the Asp residue that is the N-terminal amino acid of the mature protein, after cleavage of the signal sequence.

The construct for N355 was similar to that for the full-length protein, except that the endoplasmic reticulum-targeting signal KDEL followed by stop codons was introduced by PCR into codon 356 of the mature GRP94 sequence. N355 therefore contains the entire sequence, which is homologous to the proteolytic N-terminal domain of HSP90 (12), and in addition it contains the first acidic domain. The Δ355 construct contained amino acids 356–802 of murine GRP94. It was expressed in SF9 cells as a His-tagged fusion protein, as described above.

All the above constructs were expressed in SF9 cells via baculovirus infection, following the protocols in the manufacturer’s manual. Pellets containing 2 × 108 infected cells were lysed in 1% Nikkol (Sigma Chemicals) in 20 mM phosphate buffer pH 7.2, containing 500 mM NaCl and 20 mM imidazole. The recombinant proteins were purified from the soluble fractions of cell lysates, after centrifugation to remove nuclei and cell debris. The supernatants were passed through 0.2-mm filters and then loaded onto nitrolotriacetic acid columns (Qiagen), according to the manufacturer’s instructions. Bound proteins were eluted with 20 mM phosphate buffer pH 7.2, containing 500 mM imidazole and 500 mM NaCl, dialyzed, and concentrated. Proteins were stored in 25 mM HEPES (pH 7.2), 110 mM KOAc, 20 mM NaCl, 1 mM MgOAc2, 0.1 mM CaCl2, buffer A containing 30% glycerol. The preparations used in this work were >80% pure as determined by SDS-PAGE. In some cases, further purification by ion exchange columns was performed, but the activity of the proteins was unchanged when the purity exceeded 95%.

Tissue-derived GRP94 was purified from pooled mouse livers as described in Ref. 27. BIP was tagged with His6 and purified as described in Ref. 28.

Peptides—All peptides were synthesized at the University of Chicago facility and verified by mass spectrometry. The sequences of the peptides and their proteins of origin are as follows: VSV8, RGYYVRGL, from the VSV N protein; Pep A, KRQYTDLEMNRLGK, from the VSV G protein, NYLA, NYLAWQ4KPG, from the human immunoglobulin light chain; RAH, RAHYNITVF, from the E-6 protein of human papilloma virus 16; FYQ, FYQLAL, a synthetic pan-HSP70-binding peptide. Stock solutions were prepared in either Me2SO or water and stored at −80 °C. Peptide concentrations were determined by a BCA assay (Pierce). Peptides containing tyrosine residues were iodinated by the IodoBead method (Pierce), and unincorporated iodine was removed by passage over a short Dowex AG1X8 column. The specific radioactivity of the peptides was routinely 2 × 1014 to 10 × 1015 cpm/mol.

Peptide Binding Assays—Assays of peptide binding to GRP94 were routinely performed in 25-μl total volume of buffer A, with 2–4 μg of recombinant protein and 100–1000 μM [35S]labeled peptide. Radicicol (Sigma Chemicals; NCI program on biological response modifiers), dissolved in Me2SO, were used as inhibitors of binding. Maximal final concentration of Me2SO was 1%. In some experiments, the reactions were incubated at room temperature for up to 20 h, a time that allows saturation binding at the peptide and protein concentrations used here. In other experiments, chaperone-peptide mixtures were incubated at 50 °C for 10 min, followed by 30 min at room temperature. These two procedures yield indistinguishable data with respect to dose, specificity, or reversibility of binding. At the end of the incubations, reducing SDS sample buffer was added, and samples were resolved on polyacrylamide gels. The intensities of bands migrating as 100-kDa species (in the case of full-length GRP94) or 55-kDa species (for N355) were quantified by phosphorimaging, using a STORM 820 phosphorimager and the associated ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The unbound peptide band in the samples was also quantified, to enable the calculation of binding in absolute units. In some experiments, binding was measured by direct γ counting after separation of the bound and free peptide over spin columns containing F30 beads in buffer A. For these experiments, 20 μg of protein was used per reaction. There was excellent correspondence between the data obtained by the two methods of quantitation. The same spin column method was also used semipreparatively to separate peptide-chaperone complexes from free peptides. Peptide binding to BIP was assayed by the spin column methods described in Ref. 28.

To demonstrate saturable binding, dose binding assays of [35S]peptides were performed by adding increasing concentrations of peptide to a fixed concentration of GRP94 (2–20 μM, depending on the assay, as above). Apparent Kd values were calculated from IC50 values (the concentrations of peptide required to obtain half maximal inhibition) as described in Ref. 28. We note that these values are only apparent because of the unusually long time required for peptide dissociation. Nonetheless, they provide a means of quantitative comparison of peptides.

Gel Electrophoresis and Quantitation—Peptide-chaperone complexes were analyzed by electrophoresis through a 10% reducing SDS gel. After drying, the gels were exposed to a low energy phosphorimager screen for 2–16 h for quantification. Analysis of GRP94 or N355 by native gel electrophoresis was accomplished by using 5–15% gradient acrylamide gels in the Laemmli gel system without SDS. For blue native gels (29) Coomassie Brilliant Blue G 250 (Sigma Chemicals) was included in the cathode buffer at a final concentration of 0.02%. When dark blue bands signifying binding of the dye to protein were visible, the cathode buffer was replaced with buffer without dye, to destain the gels during the subsequent run.

In Vivo Translation and Immunoprecipitation—GRP94 constructs, FLAG-tagged at either the N or C terminus and lacking the signal sequence, were translated in vitro with a TnT kit (Promega, Madison, WI), in the absence of microsomal membranes and in the presence of [35S]Met/hom, according to the manufacturer’s instructions. Constructs for in vitro translation were made from the pGEM99.2 plasmid by addition of the FLAG octapeptide via PCR to either the N or C terminus of the GRP94 coding sequence, followed by cloning into pGEM-T Easy (Promega, Madison, WI). The products were verified by sequencing. Translation products were immunoprecipitated with M1 or M2 monoclonal anti-FLAG (Sigma Chemicals) followed by protein A-Sepharose (Reagents Corporation, Needham, MA), or 9G10 monoclonal anti-GRP94 (StressGen, Vancouver, BC) followed by protein G-Sepharose (Sigma Chemicals or Pierce) as described in Ref. 30. They were resolved by SDS-PAGE, and the gels were dried, exposed to phosphorimager screens, and recorded using the STORM 820.

RESULTS

The characterization of the mode of peptide binding by GRP94 is important because of the ability of this chaperone to mediate presentation of peptides to T cells (31). Toward this end, we have produced recombinant GRP94, using the baculovirus expression system and analyzed the peptide binding properties of both full-length GRP94 and a truncated version, consisting of the N-terminal 355 amino acids, termed N355. N355 consists of two putative domains. Its first 263 amino acids are highly homologous to the crystallized N-terminal domain of HSP90, which contains a nucleotide-binding site. The sequence 265–344 is rich in acidic residues (39%), is longer in all GRP94s than in the HSP90 proteins, and is referred to in this paper as an acidic (or negatively charged) domain. As was shown previously, this acidic domain is necessary for radicicol or geldanamycin binding to the N-terminal domain of GRP94 (15). N355 therefore contains the necessary structural determinants for at least one known activity of GRP94, binding of inhibitory ligands.

Two previously known GRP94-binding peptides, the octamer
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VSV8 (7) and the 15-mer peptide A (10), were labeled with radioactive iodine and used to assess the activity of the two recombinant proteins. The iodinated peptides were allowed to bind in solution, followed by separation of bound from free peptides using either spin columns or gel electrophoresis. As previously shown by Blachere et al. (4), the complexes between GRP94 (or N355) and peptides were stable enough to withstand resolution by SDS gel electrophoresis. Therefore, in the current study peptide binding to the recombinant proteins was quantified both by gamma counting and phosphorimaging.

**Peptide Binding to Recombinant GRP94 Occurs Via an N-terminal Site—VSV8 bound to N355 in dose-dependent fashion (Fig. 1a).** Saturation was reached at a concentration of 800 μM, and the estimated apparent Kd was 400 μM. Similar binding was observed for peptide A (Fig. 1b). Binding of VSV8 and peptide A was specific, it was inhibited by addition of excess unlabeled peptide (Fig. 2b). Significantly, peptide binding was inhibited by incubation with radicicol (Fig. 1, a and c; for peptide A see Fig. 3). This fungal metabolite is a pan-HSP90 ligand and is widely used as an inhibitor of HSP90 chaperone function (32, 33). Half maximal inhibition of peptide binding was observed at ~50 μM radicicol (Fig. 1c). We previously showed that radicicol binds to GRP94 with an estimated Kd of 0.1 μM (15), and the radicicol-binding pocket in the N-terminal domain of GRP94 (34) is known to be virtually identical to the radicicol-binding pocket identified in the crystal structure of HSP90 (32, 33). Furthermore, radicicol and geldanamycin were shown to inhibit the chaperone activity of GRP94 in vitro. Thus, peptide binding in our assay was dependent on a known structural aspect of GRP94 and is very likely to be relevant to the physiological function of GRP94.

Since a VSV8-binding site was previously mapped to the region around amino acids 624–630 of GRP94 (16), we created a construct complementary to N355, encompassing amino acids 356–802, and tested its ability to bind VSV8. This construct, termed Δ355, was expressed and purified from insect cells by the same method used for N355. As shown in Fig. 1d, when tested for binding of iodinated VSV8, Δ355 showed only marginal level of peptide association, no different from that displayed by serum albumin. Δ355 binding of the VSV8 peptide was at least 20-fold lower than that of N355 (as determined by phosphorimaging), and we consider it a nonspecific association.

Since to date there is no other known functional assay for the C-terminal portion of GRP94, we could not exclude the possibility that the lack of binding activity by Δ355 was due to inactivity or misfolding of this protein construct. We therefore compared the peptide binding ability of N355 to that of the full-length GRP94. As shown in Fig. 2a, the VSV8 binding curve of N355 was very similar to the binding curve of full-length GRP94, with saturation at 800 μM and an approximate Kd of 450 μM. The occupancy levels of both GRP94 and N355 are similar, typically 0.5–0.7 mol of peptide bound per mol of chaperone (Figs. 1 and 2) and in some assays as high as 0.9. Iodinated peptide binding to each version of the protein was inhibited by excess cold peptide, and the cold inhibition curves for both full-length GRP94 and N355 are essentially superimposable (Fig. 2b). As can be seen from comparison of the data in Figs. 1 and 2, excess cold peptide inhibited binding to the same extent as excess radicicol. Furthermore, similar cold competition assays showed that VSV8 and peptide A inhibited each other’s binding (data not shown), indicating that both peptides bind to the same site.
It was possible that recombinant GRP94 differed in its peptide binding activity from tissue-derived GRP94, previously used to assess this activity (4, 5, 7, 35). Therefore, we compared recombinant GRP94 with liver-derived GRP94 purified using the procedure of Srivastava et al. (27). In our hands, the specificity, affinity, and sensitivity to inhibitors of recombinant GRP94 were indistinguishable from that of liver-derived GRP94 (data not shown). Thus, the properties of our recombinant GRP94 are the same as those of the natural protein. We conclude that the N355 protein, containing the first two domains of GRP94, can essentially account for the known peptide binding activity of the full-length protein. Although our data do not formally exclude the possibility that GRP94 contains another peptide-binding site, they strongly suggest the existence of only a single peptide-binding site in the N-terminal portion of the chaperone.

**The Peptide Specificity of GRP94 Is Different from That of BiP**—A number of ER chaperones are known to be peptide-binding proteins. The best defined is BiP (36), but several other chaperones, including calreticulin (37), calnexin, ERP72 (38), GRP170, and protein-disulfide isomerase (39) may be active in antigen re-presentation to T cells. Because GRP94 often binds to the same protein substrates as BiP, we compared the peptide selectivity of GRP94 and BiP. The binding conditions for the two proteins are sufficiently different that a very rigorous comparison cannot be made at present, but we did establish a rank order of respective affinities among a small set of peptides. Inhibition of binding by radicicol was used as a criterion for specific binding to GRP94 (or N355), much in the same way that inhibition by ATP is used to show specific peptide binding.

**Characteristics of Peptide Binding**—As in studies by Wearsh and Nichchita (5) the peptide binding activity of GRP94 was insensitive to treatments with either ATP (Fig. 3) or ADP (data not shown) (10). This is interesting, because the adenine nucleotides and radicicol bind to the same site in HSP90 (12, 13) that is also conserved in the GRP94 sequence. The fast on/off rate of adenine nucleotides compared with the very slow off-rate of radicicol are the likely reason for this difference (see below).

When resolved by native gel electrophoresis, complexes between N355 and iodinated peptides migrated predominantly at the monomer size of N355 with a smaller fraction of complexes migrating as N355 dimers (Fig. 4a) and some higher order oligomers (not shown). The ratio of monomer to dimer in the

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**Fig. 2.** Peptide binding to recombinant GRP94. *a,* recombinant full-length GRP94 (3.6 μM) was incubated at room temperature for 20 h with increasing concentrations of iodinated VSV8 peptide and binding was quantified and presented as described in the legend to Fig. 1. Shown are mean values (± S.D.) of association in the absence (black triangles) or presence (white triangles) of radicicol (300 μM). *b,* cold competition of peptide binding. Iodinated VSV8 (600 μM) was incubated at room temperature for 20 h with either recombinant GRP94 (triangles) or N355 protein (squares) (3.6 μM each), in the absence of unlabeled peptide, or in the presence of increasing concentrations of unlabeled peptide. The extent of binding was quantified as described in the legend to Fig. 1. In the absence of unlabeled peptide the occupancy was 0.77 mol of VSV8 per mol of chaperone.

**Fig. 3.** The selectivity of peptide binding by chaperones. GRP94, N355, or BiP were loaded with either VSV8, peptide A or FYQ, under conditions that approach complete binding, as in legend to Fig. 1. Each chaperone was at 15 μM per reaction, and the peptides were at 300 μM each. The specific activities of all three peptides were similar. The binding reactions included either no inhibitor, 1 mM ATP, or 300 μM radicicol. The complexes of peptides and chaperones were then resolved by SDS-PAGE and detected by phosphorimaging. VSV8 and PepA bind to GRP94 and N355 much more avidly than to BiP, whereas FYQ binding to BiP is stronger than to GRP94 or N355. It should be emphasized that because BiP-peptide complexes are partly sensitive to SDS, unlike GRP94-peptide complexes, the data for BiP binding are underestimates of the actual levels of binding. As shown before, the reason for the incomplete inhibition of peptides binding to BiP by ATP is re-association of complexes under the conditions employed; complete inhibition is observed when a single cycle binding assay is performed (28).
autoradiograms was similar to the ratio observed by protein stains (not shown) allowing the conclusion that the N355 monomer is as competent to bind peptide as the dimer. The gel migration of N355 (3.6 μM) is slower than expected based on its actual size (44 kDa), because of its non-globular shape. The radioactivity associated with both the monomeric and dimeric N355 was decreased proportionally when peptide was allowed to bind in the presence of radicicol (Fig. 4a). In addition to verifying the previous conclusions, this analysis shows that N355 and not a contaminant is the active entity in the assay.

The time course of peptide binding was determined, to estimate the association rate constant. The on-rate was extremely slow; more than 5 h were required for detectable binding of good binder peptides at room temperature; after 24 h binding was still linear and saturation was approached only after 36 h (Fig. 4b). The slow binding was not accelerated if the protein was preincubated overnight under the reaction conditions. However, peptide binding to N355 was accelerated significantly upon heating for 10 min at 50°C, the protocol used by Srivastava et al. (27) to achieve optimal binding to tissue-derived GRP94. As much peptide bound to N355 within 30 min after this heat shock as did after overnight incubation without the heat shock; saturation levels, stability of the complexes (see below), and sensitivity of peptide binding to radicicol were not affected by heat treatment (data not shown). Therefore, heat treatment only affects the on-rate of binding, consistent with heat-induced conformational change in N355 (or GRP94) from an inactive to active conformation, a conversion that is very infrequent at room temperature or even at 37°C (data not shown).

In our hands, as in the hands of others (4), GRP94- and N355-peptide complexes were highly stable, and many conditions that usually dissociate such complexes were ineffective. To define conditions of peptide dissociation, the N355-VSV8 complexes were separated from unbound peptide via spin columns and then subjected to various treatments. The complexes were resistant to SDS, as shown above, to incubation with EDTA (Fig. 4c), ATP, and to pH 2.0 or 9.0 (data not shown). Treatment with radicicol after the binding was complete did not dissociate the peptides (Fig. 4c). Thus, radicicol decreases...
in the presence of radicicol. Evidently, the partial dissociation in the presence of urea alone observed up to this concentration (data not shown). Inclusion of chemical evidence that no significant unfolding of N355 is the top concentration of 3 M urea was chosen based on physico-

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tide were the same in the mobility of protein-peptide complexes, labeled via the pep-

col-trapped conformer. Saturation of N355 with the radioactive protein, with the faster-migrating form representing a radici-

Comparisons of various buffer conditions showed that pep-

type binding to N355 was as efficient in the buffer used by Nichitta’s group (10) as in the buffers used by Srivastava’s (4) or Sastry’s group (18), but was decidedly inferior in phosphate-buffered saline (data not shown). Binding was somewhat improved by lowering the pH below 7; conversely, it was markedly reduced at pH above 7.5 (Fig. 4d).

Peptide and Drug/Nucleotide Ligands Bind to Distinct Binding Sites on GRP94—Because radicicol prevented peptide binding, but did not dissociate GRP94-peptide complexes, we asked whether peptide and radicicol bind to the same site, or whether radicicol binding exerts allosteric regulation on peptide-bind-  

We took advantage of the inability of radicicol to dissociate preformed complexes of radioactive peptides and N355, incubated with the drug and resolved them on native gels. The mobility of protein-peptide complexes, labeled via the peptide, was shifted by subsequent treatment with radicicol, and the shift was detectable both by Coomassie Blue staining of the protein (lanes 3 and 4) and by detection of radiolabeled peptide (lanes 5 and 6). Since total counts from N355-associated peptide were the same in lanes 5 and 6, radicicol-induced migration shift did not depend on the release of peptide. A second, more direct demonstration of concomitant binding is shown in Fig. 5b. Complexes of N355 and VSV8, labeled via the peptide as above, were capable of binding to geldanamycin-conjugated beads (the conjugated beads were a generous gift of Dr. Len Neckers, NCI). Their binding was not due to dissociated free peptide, and it was competed with excess free radici-

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Ligand-induced Conformational Changes—In a second experimental approach to the relation between the binding sites for peptide and for radicicol, we examined the conformation of GRP94 as probed with monoclonal antibody 9G10. The epitope recognized by this antibody is located in the first acidic domain of the protein, within amino acids 290–350 (Ref. 41 and our own data). We extended the initial observation of Loo et al. (53) that 9G10 fails to recognize GRP94 from geldanamycin-treated cells to show that this is due to a conformational loss of the 9G10 epitope (Fig. 6). In vitro translated GRP94 typically migrated as three bands with apparent sizes of 100 kDa (the full-length mature protein), 90 and 80 kDa (two proteolytic fragments; Fig. 6). The 100-kDa and 90-kDa polypeptides, but not the 80-kDa polypeptide, bind radicicol specifically in a pull-down assay (see Fig. 8 in Ref. 15). When GRP94 was tagged with a FLAG peptide at its C terminus, all three forms were immunoprecipitable by anti-FLAG antibody (M2) (Fig. 6a, lanes 3 and 4); immunoprecipitation of the N-terminal FLAG-tagged construct (Fig. 6b, lanes 7 and 8) showed that only the full-length GRP94 retained the FLAG peptide, demonstrating that the 90- and 80-kDa polypeptides lack sequences from their N termini because of degradation in vitro. Anti-FLAG antibody was able to recognize GRP94 whether or not radicicol was added.

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Ligand-induced Conformational Changes—In a second experimental approach to the relation between the binding sites for peptide and for radicicol, we examined the conformation of GRP94 as probed with monoclonal antibody 9G10. The epitope recognized by this antibody is located in the first acidic domain of the protein, within amino acids 290–350 (Ref. 41 and our own data). We extended the initial observation of Loo et al. (53) that 9G10 fails to recognize GRP94 from geldanamycin-treated cells to show that this is due to a conformational loss of the 9G10 epitope (Fig. 6). In vitro translated GRP94 typically migrated as three bands with apparent sizes of 100 kDa (the full-length mature protein), 90 and 80 kDa (two proteolytic fragments; Fig. 6). The 100-kDa and 90-kDa polypeptides, but not the 80-kDa polypeptide, bind radicicol specifically in a pull-down assay (see Fig. 8 in Ref. 15). When GRP94 was tagged with a FLAG peptide at its C terminus, all three forms were immunoprecipitable by anti-FLAG antibody (M2) (Fig. 6a, lanes 3 and 4); immunoprecipitation of the N-terminal FLAG-tagged construct (Fig. 6b, lanes 7 and 8) showed that only the full-length GRP94 retained the FLAG peptide, demonstrating that the 90- and 80-kDa polypeptides lack sequences from their N termini because of degradation in vitro. Anti-FLAG antibody was able to recognize GRP94 whether or not radicicol was added.

All three forms of GRP94 were recognized by 9G10 in the absence of radicicol (albeit at different efficiencies), but in the presence of radicicol only the 80-kDa form was immunoprecipi-
FIG. 6. Loss of the 9G10 monoclonal epitope upon treatment with radicicol, geldanamycin or ATP. a, GRP94 tagged with the FLAG epitope at the C terminus was in vitro translated with [35S]Met/Cys. The construct used here lacked the endogenous signal sequence, and the translation was performed with reticulocyte lysate without added membranes. Left panel, equal aliquots of the reaction were immunoprecipitated with either anti-GRP94 (9G10) or anti-FLAG (M2), in the presence of 10 μM radicicol or Me2SO as a drug vehicle control. The 9G10 antibody recognized full-length GRP94 as well as two faster migrating forms with apparent mobility of 90 and 80 kDa in the absence of radicicol (left panel, lanes 1 and 2). After radicicol treatment, only the 80 kDa form was immunoprecipitated. The M2 antibody recognized all three forms of GRP94 irrespective of the presence of radicicol (lanes 3 and 4). Right panel, schematic representation of the three forms of C-terminally FLAG-tagged GRP94. All forms have the FLAG tag: the full-length molecule contains a radicicol-binding site (gray shaded box; residues 98–246) and the 9G10 epitope (hatched box; residues 280–344); the 90 kDa form lacks ~90 amino acids from the N terminus; the 80 kDa form lacks most of the Rad-binding domain. b, left panel, the 9G10 antibody did not recognize the full-length and 90 kDa forms of N-terminally FLAG-tagged GRP94 in the presence of radicicol (left panel, lanes 1 and 2). The M2 antibody recognized only the full-length form of N-terminally FLAG-tagged GRP94 independent of radicicol treatment (lanes 7 and 8). Right panel, schematic representation of N-terminally tagged GRP94; all three forms have the N-terminal truncation pattern identical to that described in a; only the full-length protein retained the FLAG tag. c, in vitro translated GRP94 (in this case, without FLAG) was incubated with ATP, ADP, or geldanamycin prior to immunoprecipitation with the 9G10. All three ligands rendered the full-length and the 90 kDa forms of GRP94 unrecognizable by 9G10, just like radicicol. The nature of the amino acids sequence preceding residue +1 of mature GRP94 has no effect on its reactivity with the antibodies used or on its ability to bind peptides (data not shown). d, recombinant GRP94 bound by radicicol is also not recognized by 9G10. In the presence of the drug, GRP94 is present only in the unbound fraction (bottom panel) and not in the antibody-bound fraction (top panel). 0.1 μM recombinant GRP94 was incubated in the absence or presence of 10 μM radicicol for 15 min prior to addition of 9G10 antibody for the immunoprecipitation. Detection was by immunoblotting with anti-GRP94 developed with the enhanced chemiluminescence reagent.

FIG. 7. Peptide-bound N355 is still recognized by antibody 9G10. Complexes of N355 with [125I]-VSV8, prepared as in Fig. 4 were purified by a spin column and incubated with and without monoclonal antibody 9G10 (+ and −, respectively). The complexes were then resolved by blue native PAGE and visualized by phosphorimaging. Samples are shown in duplicates. Note the retarded mobility of [125I]-VSV8-bound N355 in the presence of the antibody, including complexes that fail to enter the gel, indicating recognition of peptide-chaperone complexes with 9G10 antibody.

results as follows: deletion of the N-terminal 20 kDa by proteolysis abolishes the radicicol/nucleotide-binding pocket, so that the 80-kDa fragment is unable to bind either ligand and is always recognized by 9G10. A 10-kDa deletion removes N-terminal sequences up to, but not including, the ligand-binding domain (see Fig. 6a), which presumably starts at amino acid 98 (12, 26). Both the 90-kDa fragment and full-length GRP94 lose the 9G10 epitope when the ligand-binding pocket is occupied.

The loss of the 9G10 epitope was clearly demonstrated by treatments with both radicicol and ATP (Fig. 6c) and not only with in vitro translated proteins, but also with the purified recombinant proteins used in the peptide binding assays (both full-length recombinant GRP94 (Fig. 6d) and N355 (not shown). Thus, most N355 molecules do indeed bind ATP. We conclude that after binding of any of the known ligands to the N-terminal domain of GRP94 (except for bis-ANS, Ref. 11), a similar conformational change is propagated downstream to the acidic domain, leading to alteration of the 9G10 epitope.

We next asked whether binding of peptide to N355 induces a similar conformational change. When complexes of N355 with radiolabeled peptide were analyzed by blue native gels after incubation with the antibody, the radioactive label shifted from the fast and slow migrating forms of N355, this demonstrates that peptide-bound N355 was still recognized efficiently by this antibody (Fig. 7). Together with the observation that peptide binding does not shift the equilibrium between the fast and slow migrating forms of N355, this demonstrates that occupancy of the two binding sites of GRP94 has different effects on the protein conformation, with the drug-binding site controlling the accessibility of the peptide-binding site.

DISCUSSION

One significant finding of the present work is the demonstration that the peptide binding activity of the chaperone GRP94 resides within the N-terminal 355 amino acids. The N355 fragment, which encompasses two domains, is sufficient to account for the peptide binding activity of the entire protein, including binding of the immuno-dominant peptide of vesicular stomatitis virus (7, 16). Peptide binding by this fragment has the same specificity and intrinsic binding parameters as that of the whole GRP94 protein. This localization of peptide binding is consistent with a recent report that the homologous protein HSP90 binds peptides via an N-terminal site (23). It is, however, inconsistent with the data of Sastry and co-workers (16–18) who have characterized another peptide-binding site, in the
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C-terminal domain of GRP94. Our data do not exclude the possibility that there are two distinct peptide-binding sites in GRP94. After all, the related chaperone HSP90 possesses a peptide-binding site in its N-terminal portion and another protein-binding site in its C-terminal region (22, 23, 42). We cannot, however, explain our inability to detect VS8 binding activity in the C-terminal fragment of GRP94, nor why Linderoth et al. (16) did not detect the binding site that our data point to, using their cross-linking approach.

The second significant finding of our work is that peptide binding is inhibited by radicicol (and geldanamycin, data not shown), providing much needed functional specificity to the binding assay. Young et al. (22) previously showed that geldanamycin inhibits the chaperone activity of the N-terminal domain of HSP90. In this study we find that radicicol inhibits peptide binding to the corresponding domain in GRP94 by preventing the association reaction, but does not affect the dissociation stage. Given the sequence similarity between HSP90 and GRP94, further studies are therefore needed to clarify the peptide binding cycle of this family of chaperones. As we show elsewhere, radicicol is also an effective inhibitor of the chaperone activity of GRP94 in vivo. Several studies showed that the ansamycin antibiotics inhibit the function of the cytosolic HSP90 in vitro and in vivo (e.g. Refs. 22, 32, 43) by binding to a site in the N-terminal most domain (12, 33) that is conserved in GRP94 (15). Together therefore, our data suggest that the peptide binding, the protein binding, and the inhibitor binding activities of GRP94 are related mechanistically.

While the precise sites responsible for the above activities are yet to be mapped, we showed here that the peptide-binding site is not identical to the radicicol-binding site, because both peptide and radicicol can bind simultaneously to the N355 site. Not only are the peptide and radicicol binding distinct physically, their effects on GRP94 structure are also different. While radicicol binding induces a conformational change that affects the downstream acidic domain (which harbors the epitope for monoclonal antibody 9G10), peptide binding does not induce a similar conformational change. Instead, peptide binding induces a different conformational change, detected as a change in tryptophan fluorescence of the protein. In addition, radicicol binding but not peptide binding traps N355 in the fast-migrating conformation.

Work on purified GRP94 and HSP90 suggested that they exist mainly as dimers, joined tail to tail via their C-terminal domains (44 – 46). Dimers of the N-terminal domain of HSP90 have also been reported and hypothesized to form a peptide-binding site (20). As shown here, the recombinant N355 protein exists predominantly as monomers and binds peptides as monomers, although dimerization (and higher order oligomerization) neither augments nor interferes with peptide binding. In this respect, GRP94 is different from BiP/GRP78, which is converted to monomers upon peptide binding (47 – 49).

Our data lead to the following model for the action cycle of GRP94. The N-terminal half of GRP94 exists in equilibrium between a closed and an open conformation, each characterized by a distinct electrophoretic mobility in native gels (this study) and differential sensitivity to proteases. These states of N355 may correspond to the two conformations with different hydrophobic dye binding abilities observed for tissue-derived full-length GRP94 (10, 11). Although much of the protein is in the open conformation, based on the gels, we propose that it accesses the active state only from the open conformation and that it does so very infrequently under in vitro conditions.

Radicicol binding to the nucleotide-binding site of N355 converts it to the closed conformation (faster migrating band) and since radicicol binds essentially irreversibly (radicicol remains bound during overnight incubation in immunoprecipitation reaction), the protein is trapped in the closed, inactive conformation.

Binding of ligands to the N-terminal nucleotide site is not only inhibitory. Wassenberg et al. (11) have demonstrated that bis-ANS binds to the same site as radicicol, but activates peptide binding by GRP94. Like the inactivation of N355 by radicicol (this report), the activation of GRP94 by bis-ANS (11) is mediated by a conformational change. Since we have demonstrated here that the peptide-binding site is distinct from the nucleotide-binding site, it appears that ligand binding to the nucleotide-binding site exerts allosteric regulation on the peptide-binding site. This regulation is apparently unidirectional, as peptide binding itself does not affect the equilibrium between the two electrophoretic forms of N355 or the accessibility of the 9G10 epitope. Because the majority of GRP94 when isolated from either insect cells or from tissues is in the peptide binding-incompetent state, there is a need for activation of ligand binding, achieved with heat shock or mild denaturant treatment, to shift the equilibrium sufficiently and augment the peptide binding activity of the purified proteins. Heat shock treatment accelerates the interconversion at least 100-fold (36 h versus 10 – 20 min for near saturation binding without or with heat shock, respectively).

In the cell, presumably, there are factors that interact with GRP94 and either increase its conversion to an active (peptide binding) state or promote its inactivation. One potential mechanism would be phosphorylation, since Melnick (25) showed that only non-phosphorylated GRP94 binds substrate (immunoglobulin light chain in that case). Another possibility is that co-chaperones continually modulate GRP94 activity, and they are lost during purification of the protein. The cytosolic HSP90 exists in a complex with other proteins, such as p23 and FKBP52 (50, 51), and it is possible that analogous proteins are bound to GRP94 in the endoplasmic reticulum.

The present study was focused primarily on characterization of the mode and site of peptide binding and not on the nature of binding peptides. However, even from the small sample of peptides that we tested it is evident that the specificity of GRP94 binding is quite different from that of BiP and HSP70. First, a 15-mer and an octamer peptide bound with equivalent affinity. This confirms the conclusions of Srivastava and co-workers (4, 52). Second, the best HSP70 binder peptides were poor GRP94 binders. Third, the binding was inhibited by different ligands; GRP94 peptide binding was inhibited by radicicol and not by ATP, while BiP peptide binding was inhibited by ATP, but not radicicol. The different preferences for distinct peptides suggest that each chaperone may be used to augment specific T cell responses and that effective presentation of an entire peptide repertoire to the immune system may require multiple chaperones.

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