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Mapping the Major Interaction Between Binding Protein and Ig Light Chains to Sites Within the Variable Domain

David P. Davis, ² ⁶ Ritu Khurana, ² ³ ⁶ Stephen Meredith, ² Fred J. Stevens, ³ and Yair Argon ⁴ ⁶

Newly synthesized Ig chains are known to interact in vivo with the binding protein (BiP), a major peptide-binding chaperone in the endoplasmic reticulum. The predominant interactions between the light chain and BiP are observed early in the folding pathway, when the light chain is either completely reduced, or has only one disulfide bond. In this study, we describe the in vitro reconstitution of BiP binding to the variable domain of light chains (Vₐ). Binding of deliberately unfolded Vₐ was dramatically more avid than that of folded Vₐ, mimicking the interaction in vivo. Furthermore, Vₐ binding was inhibited by addition of ATP, which competed with excess unlabeled Vₐ, and was demonstrated with several different Vₐ proteins. Using this assay, peptides derived from the Vₐ sequence were tested experimentally for their ability to bind BiP. Four peptides from both β sheets of Vₐ were shown to bind BiP specifically, two with significantly higher affinity. As few as these two peptide sites, one from each β sheet of Vₐ, are sufficient to explain the association of BiP with the entire light chain. These results suggest how BiP directs the folding of Ig in vivo and how it may be used in shaping the B cell repertoire. The Journal of Immunology, 1999, 163: 3842–3850.

Proper folding of their subunits is an essential step in the biosynthesis of Ag receptors; misfolded subunits are generally incapable of assembly or subsequent expression on the cell surface. While this is true of all secreted and membrane-bound proteins, the control of proper folding is even more crucial for B and TCRs, since the variable domains of these receptors are a product of several genetic mechanisms that diversify the receptor repertoire. Given the mechanisms of somatic recombination and hypermutation, there is heightened likelihood that the resultant Ag receptors will include amino acids that are detrimental to proper folding. Thus, it is not surprising that B and TCRs require interactions with chaperones early in their biosynthesis.

The chaperone BiP⁵ was first identified based on binding to Ig heavy chain (1, 2) and shown to be the endoplasmic reticulum (ER) member of the hsp70 class of stress proteins. BiP was also shown to interact with the light chain (LC) of Ig. We have previously demonstrated that wild-type LC binds BiP transiently during its folding in the ER, and that the avidity of binding decreases as the two domains of LC fold (3). When coexpressed with a nonre-
A number of in vitro studies to define structural features of hsp70-binding peptides employed either chemically synthesized peptides (12, 24) or affinity panning of a peptide library displayed on bacteriophages (10). The binding peptides consist of either alternate aromatic or long-chain aliphatic amino acids (10), or several successive hydrophobic residues (24). The optimal peptide is seven to eight residues (25) and binds hsp70 in an extended, \( \beta \) strand conformation (26, 27). The predictive value of these studies was elegantly demonstrated by Knarr et al. (28) in mapping BiP binding sites on Ig heavy chains, and by Rudiger et al. (24) in mapping dnaK binding sites of several proteins.

In contrast to the multitude of peptide-binding studies, the interaction of BiP with natural occurring substrate proteins has scarcely been studied. Since the above-mentioned data with the dominant-negative BiP and with the point mutations in LC suggested that the predominant interaction with BiP is mediated by the Fc domain, we set out to reconstitute this interaction in vitro. Work described in this paper shows that the binding of the entire protein can be accounted for by as few as two BiP-binding peptides. The implications of these results for the progression of folding in the cell and for the selection of the LC repertoire are discussed.

Materials and Methods

Protein purifaction

**GST-BiP.** The pDS78 plasmid, encoding a GST-hamster BiP fusion protein, was obtained from Dr. H. Weissbach (Roche, Nutley, NJ) and grown in E. coli DH5a. The bacteria were induced for 3 h at mid-log growth with 1 mM isopropyl \(-D\)-thiogalactoside for 24 h.

**LEN, SMA, and REC were conjugated to 6-fluorescein-5 (and -6)-carboxamido hexanoic acid and succinimidy ester (SFX) (Molecular Probes, Eugene, OR). The FITC-conjugated peptide was separated from free FITC and unconjugated peptide by chromatography over a C-18 reverse-phase HPLC column. The fractions were collected and lyophilized.**

**Fluorescein conjugation of peptides and proteins**

The FYQLALT peptide was suspended in 100 mM borate buffer, pH 9.2 and 2 mM and coupled to FITC, as suggested by the manufacturer (Molecular Probes, Eugene, OR). The FITC-conjugated peptide was separated from free FITC and unconjugated peptide by chromatography over a C-18 reverse-phase HPLC column. The fractions were collected and lyophilized. Fresh stock solutions of labeled peptide were made in 10 mM borate buffer, pH 9, and the concentration was calculated from the absorbance at 494 and 280 nm. The F/P ratio was 1.

**LEN, SMA, and REC were conjugated to 6-fluorescein-5 (and -6)-carboxamido hexanoic acid and succinimidy ester (SFX) (Molecular Probes) by incubating at a ratio of 5 mg V\(_I\) to 2 mg SFX, according to the manufacturer’s instructions. Free SFX was separated from SFX-conjugated protein by passage through a Sephadex G-25 column (Pharmacia, Piscataway, NJ) and stored at 4°C in 0.1 M NaHCO\(_3\), pH 8.3. Incorporation of SFX yielded an F/P ratio of 3.

**Gel filtration assay for binding of peptides and V\(_I\) to BiP**

Binding assays were routinely performed in 25 mM Tris, pH 7.5, 50 mM KCl, 20 mM MgCl\(_2\), 0.5% Triton X-100, with 20 mM BiP, 42 mM \( ^{125}\)I-FYQLALT, and the appropriate concentration of unlabelled V\(_I\) or peptide for 1 h at 25°C, unless otherwise noted. When peptides were used to complete the binding, the reactions included 16% final concentration of DMSO. This concentration of DMSO in itself had no effect on the binding of \( ^{125}\)I-FYQLALT to BiP (data not shown). BiP-bound \( ^{125}\)I-FYQLALT was separated from free peptide by centrifugation for 2 min at 1000 \( \times \) g through a Bio-Gel P-30 (Bio-Rad) column. To separate BiP-bound V\(_I\) from free \( ^{125}\)I-labeled V\(_I\), similar spin columns were packed with Bio-Gel P-60 beads. The radioactivity present in the flow-through was determined by scintillation counting with a Packard Top-Count. Nonspecific binding was determined by the addition of 600-fold excess unlabeled FYQLALT, and was routinely 5–10% of the total.

**Kd values were calculated from IC\(_{50}\) values (the concentrations of peptide required to obtain half-maximal inhibition) with the Cheng and Prusoff equation:**

\[
K_d = \frac{IC_{50}}{L + IC_{50}}
\]

where \( K_d \) is the dissociation constant of the labeled ligand (derived from the dose-binding analyses, see below), \( K_d \) is the dissociation constant of the inhibitor peptides, and L is the concentration of the labeled ligand.

**To demonstrate saturable binding, dose-binding assays of \( ^{125}\)I-FYQLALT and \( ^{125}\)I-labeled V\(_I\) were performed by adding increasing concentrations of the labeled substrate to a fixed concentration of BiP (20 \( \mu \)M). Data obtained from the dose-binding analyses were fit to a Scatchard plot using the GraphPad Prism computer software to calculate \( K_d \). This program
allowed best-fit determinations of the data to either a one- or two-site binding model.

Calculation of the concentration of the BiP-LC complex (C) employed the equation:

\[ C = \frac{B + L + K_d - \sqrt{(B + L + K_d)^2 - 4BL}}{2} \]

where B is the concentration of BiP available for LC binding, L is the concentration of the LC-folding intermediates that can bind to BiP, and \( K_d \) is the dissociation constant (34).

**ATPase assays**

The extent of ATP hydrolysis was measured as previously described (35). In a final volume of 50 \( \mu \)L, 2.5 \( \mu \)g of the appropriate preparation of BiP or hsp70 (a generous gift from R. Morimoto) was incubated at 37°C in the presence of 2 \( \mu \)Ci \([\gamma-32P]ATP\). Aliquots were removed at various time points and added to activated charcoal slurry. After centrifugation, the amount of \(^{32}P\) in the supernatant was counted in a Packard Top Count.

**Gel electrophoresis analysis of the BiP-bound VL**

FITC-V\(_L\) was incubated for 1 h at 25°C with His\(_6\)-tagged KAR-2 immobilized on Ni\(^{2+}\)-agarose beads. After washing, the bound material was eluted with SDS sample buffer and resolved on a 16% acrylamide Tricine-SDS gel (36). Detection of KAR-2-bound FITC-V\(_L\) was performed by a rapid silver stain method (37).

**Prediction of BiP-binding peptides**

The distribution of putative BiP binding sites on variable domains was addressed by the use of two computer algorithms. We developed one algorithm based on the BiP-binding scores (weights) determined by Blond-Elguindi et al. (10). These scores were related to the probability of any amino acid being located at specific positions within heptameric peptides that had measurable binding to BiP. For all 20 amino acids, negative and positive numerical values were assigned to reflect the preferential exclusion or inclusion of the amino acid at positions 1–7 in BiP-binding peptides. The algorithm analyzes the variable domain sequence seven amino acids at a time by summing the position-dependent scores of each amino acid in the heptamer, assigning the resulting cumulative score to the first residue of the peptide. The algorithm was used to evaluate individual variable domains, as well as to determine an average BiP-site distribution by automatically analyzing a database of 122 human \( \kappa \) domain sequences.

**Fluorescence measurements**

Intrinsic tryptophan fluorescence was measured with 3 \( \mu \)M of V\(_L\) in 20 mM Tris, pH 7.5, and 150 mM NaCl in the absence or presence of the appropriate concentrations of Gdn-HCl or DTT (both buffered to pH 7.5). Emission spectra were recorded from 300–400 nm, using a Photon Technology Industries (Princeton, NJ) fluorescence spectrophotometer, at excitation wavelength of 280 nm. Raw data were corrected for the quenching effects of the reducing agents. Measurements of binding of the fluorescent dye ANS to the native or unfolded V\(_L\), LEN, were performed by incubating 10 \( \mu \)M ANS with 3 \( \mu \)M of the appropriate LEN in 20 mM sodium phosphate buffer, pH 7. The emission spectra were then recorded from 420–600 nm after excitation at 350 nm. The fluorescence of ANS alone was subtracted from the spectra of ANS in the presence of LEN.

**Results**

**BiP-binding assay**

Substrate binding to BiP was determined by reacting the two in solution, followed by separation of bound and free reactants. The peptide FYQLALT, which was previously shown to bind to a variety of hsp70 family members (27, 32), was labeled by iodination or by coupling to FITC, and incubated with recombinant hsp70 proteins produced in bacteria. The reactions were applied to gel filtration spin columns to separate free from bound reactants. The validity of this assay is shown in Fig. 1. Of the hsp70 proteins tested, FYQLALT bound best to human hsp70. This binding was inhibited by addition of excess unlabeled peptide and by addition of ATP (Fig. 1, A and B), consistent with the known mode of action of hsp70. The iodinated and the fluorescent versions of the peptide behaved similarly. The binding of FYQLALT to hamster BiP, mouse BiP (shown below), and yeast BiP (kar-2) was lower than to human hsp70, but in each case was effectively inhibited by excess cold peptide (Fig. 1A). Interestingly, even the GST-BiP fusion protein was active in peptide binding, although less active than the authentic BiP released by thrombin cleavage of the fusion protein.

The activity of the nucleotide-binding domain of either BiP, GST-BiP, or hsp70 was determined in two ways. Bound peptides were efficiently released by incubation with ATP in a one-cycle binding assay (Fig. 1B), in which complexes were first purified by a desalting column, incubated with ATP, and repurified. This suggested that under conditions of continuous incubation (such as those used in Fig. 1A), the observed level of peptide binding is the product of multiple cycles of binding and release. The peptides were released effectively by ATP from the various rBiP, despite their low inherent hydrolytic activity as compared with the recombinant hsp70 (Fig. 1C). This is consistent with the notion that nucleotide exchange, not hydrolysis, was important for peptide release of all members of the hsp70 family of chaperones (22).

When we measured, instead of rates of ATP hydrolysis, the extent of stimulation of ATPase activity by FYQLALT, both human hsp70 and hamster BiP were stimulated 3–4-fold (data not shown).

The peptide-binding assay was used to measure the affinity of \(^{125}\)I-FYQLALT to BiP (Fig. 1D). Binding approached saturation at 500 \( \mu \)M peptide, and Scatchard analysis showed a biphasic binding curve, with a calculated \( K_d \) of 2 ± 0.8 \( \mu \)M for the higher affinity site, and a \( K_d \) of 163 ± 22 \( \mu \)M for the lower affinity site (n = 4). Since at saturation binding capacity approached a ratio of 1 mol of peptide bound for every mole of BiP, this indicates the biphasic binding is a manifestation of high and low affinity states for the single binding site of each BiP molecule (15, 38, 39). A similar observation was made with the biphasic binding of FYQLALT by the related chaperone hsp70 (32). Competition-binding assays (see Materials and Methods), in which increasing concentrations of unlabeled FYQLALT were used to displace the binding of a constant concentration of \(^{125}\)I-FYQLALT, gave a calculated \( K_d \) of 5 ± 2 \( \mu \)M for the high affinity site (Fig. 2A). The correspondence between the \( K_d \) values of the direct binding and the competition assays validates this spin column assay and shows that iodination of the tracer peptide did not significantly change its binding affinity. Therefore, it was used to measure either direct binding of labeled substrates, or the ability of unlabeled substrates to displace the labeled ones. Because GST-hamster BiP and His\(_{6}\) mouse BiP were found to exhibit equivalent binding affinities and specificities, these rBiP forms were used interchangeably in all subsequent experiments.

**Binding of V\(_L\) domains of LC to BiP**

The LEN \( \kappa \)-chain is a highly soluble Bence Jones protein isolated from a patient who excreted it at a rate of 50 g/day (31). In contrast, the SMA and REC \( \kappa \)-chains were derived from patients diagnosed with light chain amyloidosis. Recombinant LEN and SMA, in the form of V\(_L\) polypeptides produced as periplasmic -chains: LEN is stable and manifests the same monomer-dimer equilibrium typical of purified L chains, while SMA and REC are less stable proteins (31). Recombinant LEN and SMA (as well as REC, not shown) were tested for their ability to bind rBiP in a cold inhibition assay and in a direct binding assay (Fig. 2, A and B). Each protein bound best if it was first deliberately denatured (Fig. 2, compare A with C). Either treatment with Gdn-HCl or controlled reduction and subsequent alkylation gave rise to populations of unfolded V\(_L\) that bound BiP. The highest level of binding was observed when LEN, SMA, or REC was both denatured and reduced. That such treatments indeed caused denaturation of
hsp70 protein with 125 I-FYQLALT were purified over a spin column, in-
tide. BARS 125 I-FYQLALT bound in the presence of 100-fold excess unlabeled pep-
FIGURE 1. Differential peptide binding and ATPase activity of hsp70
proteins. A, The ability of human hsp70, hamster BiP, GST-BiP fusion protein, and yeast Kar2 to bind 125 I-FYQLALT was compared. Open bars = 42 μM 125 I-FYQLALT + 20 μM hsp70 or BiP; solid bars = 125 I-FYQLALT bound in the presence of 100-fold excess unlabeled peptide. B, ATP-induced release of bound peptide. Complexes of the indicated hsp70 protein with 125 I-FYQLALT were purified over a spin column, incubated for 10 min with (filled bars) or without (open bars) 10 mM ATP, and repurified over a spin column. The amount of radioactive peptide remaining in the complex was measured and is presented as relative binding.

V_L was shown by increases in the intrinsic Trp fluorescence and binding of the hydrophobic dye ANS (Fig. 2, E and F). On the other hand, the structure of BiP itself was not affected at the concentrations of denaturant present in the binding assay (<0.4 M), as judged by the same two criteria (data not shown). Direct binding of 125 I-labeled, denatured, and reduced V_L was saturable, with 88 ± 24% of BiP bound at saturation (Fig. 2B, n = 4). The calculated K_a for LEN, SMA, and REC was similar, 3–11 μM (Fig. 2, A and B, and Table I). The nature of the label had no significant effect, as similar results were observed with either iodinated or FITC-conjugated V_L (data not shown) and the binding of either form of labeled V_L was inhibited by unlabeled, denatured V_L.

No binding of V_L to BiP was detected at 25°C, as demonstrated by the inability to compete for BiP binding with 125 I-FYQLALT (Fig. 2C). Interestingly, marginal binding of all three V_L could be consistently detected in the absence of denaturant when the incubation was performed at 37°C, resulting in an apparent K_a of 300 μM at best (Fig. 2C). Analysis of V_L eluted from BiP showed that the V_L preparations contained a subpopulation that was qualita-
tively different from the majority of molecules: the BiP-bound V_L exhibited a slower mobility than the input material in Tricine-SDS nonreducing gels. A representative experiment is shown in Fig. 2D, with either native or Gdn-HCl-denatured REC that was incubated at 37°C with BiP. The mobility of the resulting BiP-bound fractions was the same, i.e., slower than the input REC. Similar results were observed with LEN and SMA (data not shown). We surmise, therefore, that a small fraction of the V_L population, as isolated from the bacterial periplasm, is nonnative and leads to the low BiP-binding activity of folded V_L. It is likely that this fraction of the protein is larger at 37°C and becomes even larger under unfolding condition. We estimate from the saturation-binding condi-
tions that at least one-half of LEN or SMA can become unfolded enough to interact with BiP under the conditions used.

Kinetic analysis of intrinsic Trp fluorescence of the denatured and reduced LEN showed that it did not diminish substantially upon incubation in the binding buffer (Fig. 2E). In contrast, sig-
nificant reduction in Trp fluorescence was observed upon dilution of Gdn-HCl-denatured LEN (with an intact disulfide bond) into the binding buffer (Fig. 2E). This suggests that the form of LEN used as a substrate in these assays did not refold significantly under the binding conditions, whereas in the case of denatured LEN, whose disulfide bond is oxidized, the fraction of the protein capable of BiP binding decreased with time, mimicking a folding reaction. Thus, just as seen in vivo, unfolded forms of V_L have higher af-
finity for BiP than the more mature, folded species.

Identifying potential BiP binding sites in V_L

To map BiP binding sites in V_L, we searched the sequence for peptides conforming to published motifs that predict likely binder

C. The kinetics of ATP hydrolysis by four recombinant hsp70 proteins. The ability of bacterially produced proteins to hydrolyze [γ-32 P]ATP was mea-
sured as the radioactivity that fails to bind to activated charcoal slurry, as described in Materials and Methods. A GST-rab fusion protein was used as a negative control for both peptide binding and ATP hydrolysis. D, Binding analysis of 125 I-FYQLALT to BiP. 125 I-FYQLALT at increasing concentrations was incubated in duplicate for 1 h at 25°C with a constant concentration of BiP. Shown is a representative experiment utilizing 5 μM His-mouse BiP; equivalent results were obtained with 20 μM GST-hamster BiP. Bound peptide was separated from free, as described in Materials and Methods. Data obtained from the dose-binding analysis (inset) were fit to a Scatchard plot using the GraphPad Prism program, to calculate K_a. Peptide binding at higher input concentrations could not be reliably determined due to peptide insolubility.
peptides. Based on the data of Blond-Elguindi et al. (10), derived from BiP panning of dodecameric and heptameric peptide libraries displayed by phages, a computer program was written to search any V\textsubscript{L} sequence. A negative score by this program indicates that the peptide has a low probability to bind BiP, while scores above +5 indicate a significant probability of binding BiP (10). Knarr et
Comparing affinities of peptides for BiP binding

To test the above predictions, 10 different V<sub>L</sub> peptides were synthesized (Table I). Some peptides were derived from the regions predicted to bind BiP (Fig. 3), while others had a fairly low predicted binding probability. The low probability peptide 61–67 was chosen, because previous data from our lab showed that point mutations in this sequence actually enhanced LC binding to BiP in vivo (7, 8). All 10 peptides were tested for their ability to compete with the binding of the labeled peptide FYQLALT to BiP. Examples of the peptide competition assays are shown in Fig. 3C, and the experiments are summarized in Table I. The apparent dissociation constants of the BiP-binding peptides that are generated by somatic mutation are listed in Table I. A similar strategy was used to test predicted BiP-binding peptides in an Ig heavy chain. Our analysis of the LEN sequence, which is a germline κV with one somatic mutation, shows multiple heptameric peptides with the requisite score (Fig. 3A).

More recently, a different motif for peptides that bind the bacterial homologue of BiP, dnaK, was defined via an extensive heptamer scan (24). This prediction may be applicable to BiP, because the related peptide PKLLIYWA was predicted to be the most likely to bind dnaK, based on the data in (24). The related peptide PKLLIYAA, which is the commonly occurring sequence in families other than κV, could not be tested reliably due to its insolubility. Using the IC<sub>50</sub> values, the K<sub>d</sub> was calculated for each peptide or unfolded V<sub>L</sub> that competed with the binding of <sup>125</sup>I-FYQLALT to GST or His-BiP. The values shown are averages of at least three experiments per peptide, except for the peptide TDFTLTI, which was tested twice.

Substituting Thr<sup>16</sup> with Tyr did not alter the measured binding to BiP. NA, not applicable.

Peptide derived from V<sub>L</sub> are listed with amino acid numbers noted in parenthesis. The predicted BiP score was calculated using a computer algorithm based on data by Blond-Elguindi et al. (10). The peptide PKLLIYWA was predicted to be the most likely to bind dnaK, based on the data in (24). The related peptide PKLLIYAA, which is the commonly occurring sequence in families other than κV, could not be tested reliably due to its insolubility. Using the IC<sub>50</sub> the K<sub>d</sub> was calculated for each peptide or unfolded V<sub>L</sub> that competed with the binding of <sup>125</sup>I-FYQLALT to GST or His-BiP. The values shown are averages of at least three experiments per peptide, except for the peptide TDFTLTI, which was tested twice.

Table I. Binding of V<sub>L</sub> peptides to BiP<sup>a</sup>

<table>
<thead>
<tr>
<th>Peptide Sequence (position in V&lt;sub&gt;L&lt;/sub&gt;)</th>
<th>Predicted BiP Score</th>
<th>K&lt;sub&gt;d&lt;/sub&gt; (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FYQLALT (11–17)</td>
<td>+11</td>
<td>5 ± 2</td>
</tr>
<tr>
<td>LAVSLGE</td>
<td>+2</td>
<td>220 ± 108</td>
</tr>
<tr>
<td>NTLAWYQ (31–37)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+12</td>
<td>41 ± 16</td>
</tr>
<tr>
<td>TLAWYQQ (32–38)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>+1</td>
<td>102 ± 38</td>
</tr>
<tr>
<td>WYQKPQG (35–41)</td>
<td>+9</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>YQOKPQG (36–42)</td>
<td>0</td>
<td>&lt;1000</td>
</tr>
<tr>
<td>PKLLIYWA (45–52)</td>
<td>+7</td>
<td>187 ± 99</td>
</tr>
<tr>
<td>DRFSGSG (60–66)</td>
<td>+2</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>RFSGSGS (61–67)</td>
<td>0</td>
<td>1000</td>
</tr>
<tr>
<td>TDFILTI (69–75)</td>
<td>+6</td>
<td>81 ± 1</td>
</tr>
<tr>
<td>FITLTISS (71–77)</td>
<td>+7</td>
<td>47 ± 15</td>
</tr>
<tr>
<td>Denatured V&lt;sub&gt;L&lt;/sub&gt;</td>
<td>NA</td>
<td>6 ± 5</td>
</tr>
</tbody>
</table>

a Peptides derived from V<sub>L</sub> are listed with amino acid numbers noted in parenthesis. The predicted BiP score was calculated using a computer algorithm based on data by Blond-Elguindi et al. (10). The peptide PKLLIYWA was predicted to be the most likely to bind dnaK, based on the data in (24). The related peptide PKLLIYAA, which is the commonly occurring sequence in families other than κV, could not be tested reliably due to its insolubility. Using the IC<sub>50</sub> the K<sub>d</sub> was calculated for each peptide or unfolded V<sub>L</sub> that competed with the binding of <sup>125</sup>I-FYQLALT to GST or His-BiP. The values shown are averages of at least three experiments per peptide, except for the peptide TDFTLTI, which was tested twice.

b Substituting Thr<sup>16</sup> with Tyr did not alter the measured binding to BiP. NA, not applicable.
another BiP binding site, peptide 71–77, forms part of which packs against the internal disulfide bond (Fig. 4, top). It is buried in the core of the native protein and includes Trp 35, a standard. Of the 10 V_L peptides, 4 did not bind BiP, including both of the two predicted binder peptides from the second region, 69–77 SGSG and RFSGSGS. Two heptamers from region I in Fig. 3, 31–41 bound slightly better than the heptamer 31–37 and considerably better than the 32–38 heptamer (Table I) is consistent with this idea. Alternatively, it is possible that each V_L is bound at more than one site and that binding of one BiP molecule to one site increases the likelihood of another BiP binding to the second site. Experiments designed to detect such possible cooperativity are in progress.

**Discussion**

This work describes the in vitro reconstitution of interactions between Ig_L chain and one of its known in vivo chaperones, BiP. This reconstitution mimics the in vivo binding specificity of BiP for the immature forms of L chain and the sensitivity of binding to the nucleotide state of BiP. The reconstituted binding was then used to map binding sites within the LC, leading to the conclusion that as few as two peptides are sufficient for this interaction.

One mechanism that is often envisioned for BiP binding to newly synthesized LC is attachment of multiple BiP molecules to appropriate peptide sites displayed along the length of the unfolded polypeptide. Data for the related hsp70 family protein dnaK show that in various random proteins, sites are found on average every 36 amino acids (24). If these data are extrapolated to BiP, there can be six binding sites per LC. However, this number is almost certainly an underestimate, because unlike the collection of substrate proteins used to generate the data for dnaK, LC consists entirely of β sheets, with no α helices, and BiP binds to peptides in the extended β conformation (26). Attachment to multiple sites along the polypeptide is also inferred from the mode of action of BiP or mitochondrial hsp70 in the translocation of proteins across membranes: the power stroke of hsp70 is proposed to mediate vectorial transport across the membrane, either as a ratchet mechanism or as a mechanochemical motor (41–43).

An alternative mechanism for the action of BiP in protein folding is attachment to key peptides that must not be left exposed lest folding take an unproductive pathway. According to this view, there are a few dominant binding sites among the many potential sites. Previous work from this lab and others (44–46) pointed to the V_L domain as the major determinant in the association of LC with BiP during folding in the cell. In this study, we present direct evidence that the V_L domain indeed binds BiP in vitro, and we map two likely dominant BiP binding sites.

The data presented in this study demonstrate that two V_L proteins, LEN and SMA, bind BiP in vitro with similar affinities. Furthermore, their binding is a property of partially unfolded intermediates and not of the native V_L, as shown by the vastly increased affinities after denaturation of the recombinant proteins. This feature recapitulates the in vivo behavior of newly synthesized LC that only associate with BiP as long as they have not been exposed to the activity of the cell's BiP, which is consistent with the idea that during translation, BiP binds to nascent polypeptides. The presence of BiP on nascent polypeptides has been previously suggested by electron microscopy studies (47) and is consistent with the observation that BiP can bind to a wide range of nascent polypeptides and can also bind to some pretranslated polypeptides (48). This binding is thought to be mediated by the presence of unexposed hydrophobic residues that are not accessible in the folded state (49).

**FIGURE 4.** BiP binding sites within V_L. A schematic representation of LC folding, adapted from (23), with the identified BiP-binding peptides highlighted. The β sheets in both V_L and C_L domains are shown in gray, and several key amino acids in V_L are indicated by their residue number, for orientation. The disulfide bonds within each domain are shown in black. The two highest affinity BiP-binding peptides are shaded black, and the two intermediate affinity sites are shaded dark gray.
Our in vitro studies identified four BiP-binding peptides in the sequences of LEN and SMA, out of the collection of predicted peptides. Of these four peptides, two bind BiP with affinities that are reasonably close to the affinity of the entire \( V_L \) protein. These two peptides are in \( \beta \) strands that are buried in the hydrophobic core of the folded \( V_L \) and are well conserved among LC sequences. One site is in strand E of the four-stranded \( \beta \) sheet, and a second site is in strand C of the five-stranded \( \beta \) sheet (Fig. 4). We propose that binding of BiP to these two peptides is sufficient to account for binding of the entire protein. Binding of BiP to one or both of these dominant sites could maintain the two halves of the \( \beta \) sandwich in an open conformation, with Cys\(^{23}\) and Cys\(^{88}\) in a reduced state. Three lines of evidence are consistent with this interpretation. First, maximal binding to BiP occurred when \( V_L \) was not only denatured, but also reduced. Second, coimmunoprecipitation data from metabolically labeled cells enabled an estimation that 2–4 mol of BiP were bound per mole of LC (3). Third, such binding accounts for the in vivo observation that prolonged association with BiP retards the oxidation of the \( V_L \) domain (4).

The binding to peptide 11–17 is intriguing: in the native state, this peptide forms a tight turn between strands A and B. This turn is a conserved feature of the Ig fold, and mutations disrupting this turn are associated with various aggregation-prone LC (Ref. 46 and footnote 6). If the binding of this peptide to BiP persists, the formation of the four-stranded \( \beta \) sheet is expected to be delayed, leaving the \( V_L \) domain in a very unfolded state. More likely, given the low affinity of this site, it may be the first of the peptides to fold, enabling the folding of the two \( \beta \) sheets, while BiP binding to the other two sites delays the formation of the disulfide bond between Cys\(^{23}\) and Cys\(^{88}\). Like the 11–17 peptide, the 45–50 peptide, whose binding affinity in vitro is intermediate, is largely solvent exposed even in the native structure. In most \( \kappa \) families, this peptide is P(K,R)LILY, a sequence that is predicted (24) to bind BiP even less avidly than the PKLLIKY sequence found in the LEN and SMA proteins. We therefore propose that the 11–17 and 45–50 peptides are not the important BiP-binding sites in vivo.

The presence of two dominant BiP binding sites in the \( V_L \) domain does not rule out the possibility of other BiP binding sites in the \( V_L \) domain or in the \( C_L \) domain. We predict, however, that because \( C_L \) folds and oxidizes even in the presence of a nonreleasing BiP (4), only low affinity BiP-binding peptides exist in this domain. The data presented in this study suggest that binding of BiP to a newly synthesized polypeptide in the cell should not necessarily be viewed as a process of coating a string of amino acids with multiple BiP molecules. Such binding to multiple low affinity peptides may mediate nascent chain translocation across the ER membrane, one of the physiological functions of BiP (47). This role is analogous to that of mitochondrial hsp70 in translocation of proteins across the organelle (42, 48). Instead, we suggest that once the proteins are in the lumen of the ER, association of BiP during folding can be explained by selective binding to higher affinity sites identified in this work.

How much of the LC in the ER is bound to BiP? The concentration of the incompletely folded intermediates of LC within the ER is 0.36–0.9 \( \mu M \) (49, 50). The total BiP concentration is estimated at 100 \( \mu M \) (49), and it is possible that only a fraction of the total pool is available to bind LC. Together with the \( K_a \) values derived in this study, it can be calculated that 32–50% of the LC intermediates should be bound to BiP when ATP is present, and 48–92% when ATP is depleted. These calculations are consistent with our data showing that only ~1/3 of the total LC can be immunoprecipitated with BiP (51), particularly considering that some substrate is likely to dissociate during the immuno-isolation procedure. The fraction of BiP-bound LC is also consistent with the kinetics of LC folding: given that LC bound to BiP cannot complete its folding, between 10% and 14% of the F\(_1\) intermediate (the one disulfide intermediate) (4) must be free at any given time to account for the rate constant of its conversion to the fully oxidized form (derived from the \( t_{1/2} \) of the F\(_1\) intermediate and assuming first order reaction).

When the human germline \( V_k \) segments are examined, the sequences of the two peptides with the highest affinity for BiP are highly conserved. The hydrophobic residues in the 71–77 sequence are invariant, and the variability is limited to a Lys-for-Thr\(^{27}\) substitution in the \( \kappa \) family, Arg-for-Ser\(^{77}\) in the \( \kappa \) and two \( \kappa \)II genes, and a few other genes with Asn instead of Ser\(^{76}\) or Ser\(^{77}\). In the 31–37 sequence, Trp\(^{35}\) is invariant, as is the tetrapeptide WYQQ, except for the \( \kappa \)I family again, displaying a few conservative substitutions (Phe or Leu for Tyr\(^{36}\); Leu for Gln\(^{37}\)). Position 34 is almost always occupied by a residue with a small side chain (Ala, Gly, or Ser) and occasionally Asn or Asp, while position 31 is always either Ser or Asn. This sequence conservation of the BiP-binding peptides is not due to protection from the somatic mutation mechanism: a number of mutations within the sequences have been identified in LC transcripts from Peyer’s patch B cells, showing that mutations in these residues do occur (52) (C. Milstein, personal communication). Therefore, the conservation is due to selection at the level of the expressed protein, presumably because these peptides are important in the proper folding of the domain.

BiP binding to framework peptides is most likely an important, yet little appreciated, selective force that participates in shaping the expressed repertoire of Ig V genes. Proper folding of variable domains depends heavily on an extensive network of hydrogen bonding (53), so many somatic mutations disrupt entire constellations of amino acids. Even mutations in the hypervariable loops are known to have global destabilizing effects on the whole domain (54). Therefore, mutations in many positions could lead to persistent exposure of the conserved BiP-binding peptides, causing prolonged BiP-LC interactions. In addition, somatic mutations may also create new, high affinity, BiP-binding peptides causing prolonged interactions. If the overall avidity for BiP is not too high, the mutant LC may actually benefit from the shielding from water and other proteins provided by BiP, manage to fold, and be included in the expressed repertoire. If, on the other hand, the mutation is catastrophic in terms of proper folding, the persistent association with BiP could delay folding, inhibit Ig assembly, and ultimately target somatic mutants for degradation. Finally, occupancy of BiP by mutant proteins is most likely used in an ER-to-nucleus signal transduction pathway, termed the unfolded protein response, and characterized in yeast (55). Thus, BiP could be a sensor that monitors V region diversity and reports back to the genetic apparatus whether to undergo further gene rearrangements.

In conclusion, our reconstitution of BiP-substrate interactions strongly supports the hypothesis that during folding in vivo BiP binds to only few higher affinity sites per substrate that are located in important elements within the folding module. By binding to them, BiP retards the folding, thus helping to ensure progress along the productive pathway. This binding mechanism is a sophisticated tool that may be used by B lineage cells to monitor the outcome of gene rearrangements and somatic mutations during the diversification of the Ig repertoire.

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