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Affinity Panning of a Library of Peptides Displayed on Bacteriophages Reveals the Binding Specificity of BiP

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Summary

We have used affinity panning of libraries of bacteriophages that display random octapeptide or dodecapeptide sequences at the N-terminus of the adsorption protein (pIII) to characterize peptides that bind to the endoplasmic reticulum chaperone BiP and to develop a scoring system that predicts potential BiP-binding sequences in naturally occurring polypeptides. BiP preferentially binds peptides containing a subset of aromatic and hydrophobic amino acids in alternating positions, suggesting that peptides bind in an extended conformation, with the side chains of alternating residues pointing into a cleft on the BiP molecule. Synthetic peptides with sequences corresponding to those displayed by BiP-binding bacteriophages bind to BiP and stimulate its ATPase activity, with a half-maximal concentration in the range 10–60 μM.

Introduction

The molecular chaperone BiP, the sole member of the hsp70 protein family localized in the endoplasmic reticulum (ER) of eukaryotic cells (for review see Gething and Sambrook, 1992), is required for translocation of newly synthesized polypeptides across the ER membrane (Vogel et al., 1990; Nguyen et al., 1991; Sanders et al., 1992) and for their subsequent folding and assembly in the ER lumen (Schonberger et al., 1991; Gething and Sambrook, 1992). The role of BiP as a chaperone depends on its ability to recognize a wide variety of nascent polypeptides that share no obvious sequence similarity, while accurately discriminating between properly folded and unfolded structures. In vitro, binding of some, but not all, short synthetic peptides stimulates the ATPase activity of BiP (Flynn et al., 1989; 1991) and alters its oligomeric state (Blond-Elguindi et al., 1993). The affinities for BiP of a small set of randomly chosen synthetic peptides varied over a 1000-fold range, but no obvious correlation was apparent between the sequences of these peptides and their association constants (Flynn et al., 1989). Bulk sequencing of a population of water-soluble heptameric peptides eluted from BiP with acid showed them to be enriched in aliphatic amino acids, particularly at positions 3–6 (Flynn et al., 1991). Unfortunately, because of the limitations imposed by the methods used, no data were available for the amino acid composition of position 1 of the peptide population, nor for the abundance of glycine (Gly), cysteine (Cys), or tryptophan (Trp) at any position, and it was impossible to identify the sequences of individual peptides whose affinities for BiP could be directly measured.

We chose to investigate the characteristics of peptides that bind to BiP using affinity screening of large, highly diverse libraries of peptides expressed on the surface of bacteriophage fd particles. This powerful technique (for review see Scott and Smith, 1990; Dower, 1992; Scott, 1992; Smith, 1991) is based on the ability of filamentous bacteriophages to display foreign peptides on their outer surfaces and involves the specific screening and affinity purification of bacteriophages displaying peptides that are ligands for a particular protein (Parmley and Smith, 1988). Peptides fused to the N-termini of the four to five copies of the pIII protein of bacteriophage fd are displayed at one tip of each bacteriophage particle and have little or no effect on viral infectivity (Scott and Smith, 1990; Dower, 1992; Devlin et al., 1990). Bacteriophage display has the following advantages over studies with random synthetic peptides: the peptides are fused to the bacteriophage proteins, minimizing peptide solubility problems; the sequences of individual peptides can be easily deduced by DNA sequencing of the appropriate coding region of the bacteriophage genome, an approach that avoids technical problems encountered during amino acid sequence analysis, such as the inability to analyze certain residues or positions of the peptide; and peptides corresponding to individual bacteriophage-displayed sequences can be synthesized and their activities assayed directly.

To study the binding specificity of BiP, we utilized two bacteriophage libraries with random octapeptide or dodecapeptide inserts at the N-terminus of the pIII protein. Affinity panning of the octapeptide library with immobilized murine BiP yielded a population of bacteriophages that display peptides whose sequences, when compared with those of peptides displayed by unselected bacteriophages, define a heptameric consensus motif that can be used to predict BiP-binding sites in natural proteins. Heptameric sequences conforming to the consensus motif were present within the dodecapeptides displayed on bacteriophages independently selected from the second library and within synthetic peptides known to bind to BiP (Flynn et al., 1989; Oblas et al., 1990). The diversity of sequences of BiP-binding peptides is consistent with the ability of BiP to interact with a wide variety of unrelated

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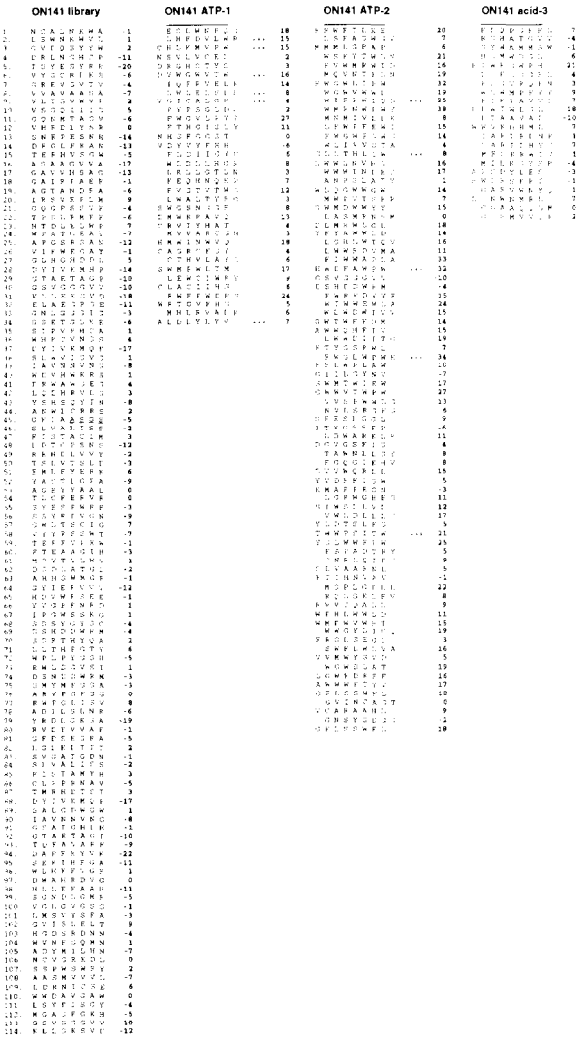

Figure 1. Amino Acid Sequences of Octapeptides Displayed by ON141 Bacteriophages Picked before or after Selection for Ability to Bind to BiP

Single-stranded DNA was purified from 114 bacteriophage clones from the original ON141 library, from 34 ATP-1 and 80 ATP-2 clones were obtained by ATP elution after one or two rounds of affinity panning in the presence of BiP, and from 22 acid-3 clones were obtained by acidic elution after three rounds of affinity panning. The sequences of the octapeptides displayed by these bacteriophages were deduced from the DNA sequence of the corresponding region of the bacteriophage genome. Many of the sequences of the octapeptides from BiP-binding bacteriophages have been shifted by one amino acid position to provide the optimal alignment that reveals the heptameric BiP-binding motif in positions defined as numbers 2–8. This final alignment was achieved by an iterative procedure. The first alignment was done such as to maximize the frequency of occurrences of Trp, Phe, and Leu residues in the even-numbered positions in the 114 ATP-1 and ATP-2 peptides. Scores such as those shown below the histogram columns in Figure 4 were assigned to each of the possible 20 amino acids at each position in the seven residue core sequence based on the relative frequencies with which particular amino acids occur at particular positions in BiP-binding heptapeptides and in randomly selected heptapeptides. This scoring matrix was used in conjunction with the BiP Score program (see Experimental Procedures) to calculate the overall score for each of the two seven residue sequences present in the ATP-1 and ATP-2 peptides, and the few peptides whose alternative alignment gave a higher score were identified. After adjusting the alignment of these peptides, the numbers of each of the amino acids at positions 2–8 were recounted, the scoring matrix was updated, and the scores were recalculated. After two rounds of this process, every peptide was aligned such that the core heptapeptide sequence (overlined at the top of the columns) gave the largest of the two scores possible for the octapeptide. For each peptide, this larger score is listed. Peptides displayed by bacteriophages that were stained most intensely in the nitrocellulose filter assays are marked with a triple plus sign.

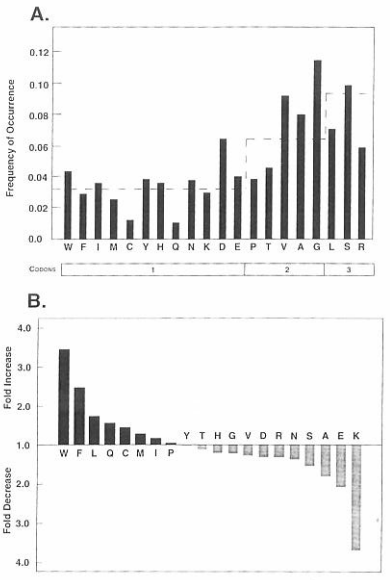

Figure 2. Relative Abundance of the 20 Amino Acids in Octapeptides Displayed by the Library of ON141 Bacteriophages

(A) Single-stranded DNA was purified from 114 bacteriophage clones from the ON141 library, and the sequences of the octapeptides displayed by these bacteriophages were deduced from the DNA sequence of the corresponding region of the bacteriophage genome. The figure shows the frequency of occurrence of each amino acid calculated as the number observed divided by the total number of residues in the 114 octapeptides. The amino acids are grouped according to the number of codons that specify them (Cwirla et al., 1990), and the frequency expected for each group if all codons were utilized with equal efficiency is shown by the dotted line. (B) The overall amino acid composition of 114 octapeptides from BiP-binding ON141 bacteriophages was compared with that of the same number of octapeptides from clones picked randomly from the ON141 starting library. The fold increase or fold decrease in the abundance of each amino acid in the selected versus unselected populations is shown. The results for Gln and Cys are less certain because the poor representation of these residues in the starting library (A) reduces the reliability of the data.

nascent polypeptides; their marked hydrophobicity is consistent with the likelihood that BiP interacts with sequences normally located in the interior of a fully folded protein.

Results

A Library of Bacteriophages That Display Octapeptides at the N-Terminus of the pIII Adsorption Protein

Because peptides containing at least seven or eight residues are required for efficient binding to BiP and maximal

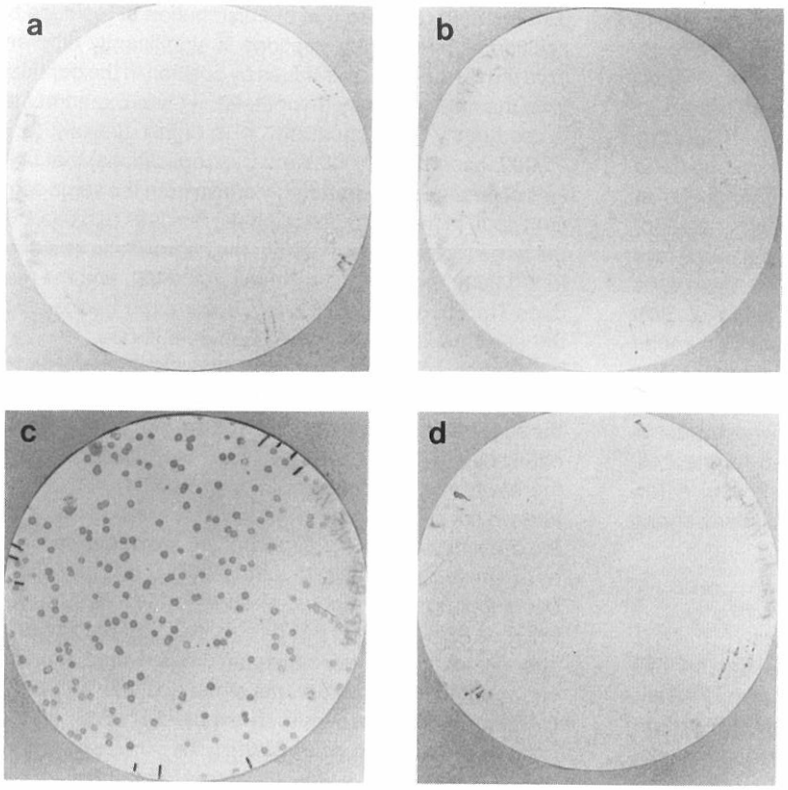

Figure 3. Selection and Screening of ON141 Bacteriophages That Express Octapeptides Recognized by BiP

Tetracycline-resistant clones of E. coli K91 cells infected with ON141 bacteriophages were adsorbed onto nitrocellulose filters. The cells were washed off the filters, and the positions of colonies secreting BiP-binding bacteriophages were revealed by incubation of the filters with biotinylated BiP, then with streptavidin conjugated to alkaline phosphatase, and then with the chromogenic substrate nitroblue tetrazolium and 5-bromo-4-chloro-3-indolylphosphate toluidinium as described in Experimental Procedures. The four filters shown originally contained 588 colonies infected with recombinant bacteriophages from the unselected ON141 library (a), 73 colonies infected with bacteriophages obtained by elution with ATP following one round of panning in the absence of BiP (b), 226 colonies infected with bacteriophages obtained by elution with ATP following one round of panning in the presence of BiP (c), and 1012 colonies infected with bacteriophages obtained by elution with HCl following one round of panning in the presence of BiP (d).

stimulation of its ATPase activity (Flynn et al., 1991), we chose initially to pan a library of bacteriophages that display octameric peptides. This library (denoted ON141) consists of 1.4×10^9 independent recombinants, each displaying a different octapeptide at the extreme N-terminus of the pIII protein. To characterize these peptides, 114 individual bacteriophage clones were picked at random from the unselected library, and the amino acid sequences of the variable octapeptide inserts were deduced from the nucleotide sequences of the corresponding coding region (Figure 1, first column). All amino acids are represented, although their frequencies do not always correspond to those expected from the relative numbers of codons encoding each residue (Figure 2A). The distribution of the majority of the residues does not vary greatly with position. However, some residues (leucine [Leu], Gly, aspartic acid [Asp], glutamic acid [Glu]) tend to be somewhat more abundant toward the N-terminal ends of the peptides, while the reverse is true for lysine (Lys) (see Figure 4).

Selection of Bacteriophages Displaying Peptides That Bind to BiP

Murine BiP expressed in Escherichia coli was purified as described previously (Blond-Elguindi et al., 1993). Biotinylation of an average of 1.5 Lys residues per BiP molecule did not significantly affect the ATPase and peptide binding activities of the protein (data not shown). The biotinylated protein was immobilized on the surface of streptavidin-coated plastic wells and incubated with ON141 bacteriophages (1×10^{12} tetracycline resistance-transducing units, ~800 library equivalents). After removal of unbound bacteriophages by extensive washing, the bound bacteriophages were eluted either by incubation in buffer containing ATP or by treatment at low pH. ATP hydrolysis promotes the release of (poly)peptide substrates bound to BiP (Munro and Pelham, 1986; Kozutsumi et al., 1989; Flynn et al., 1989) and should provide a specific method of recovery of bacteriophages displaying authentic BiP-binding peptides. Acidic elution has been the standard procedure in bacteriophage panning experiments (Parmley and Smith, 1988) and was used by Flynn et al. (1991) to elute synthetic peptides bound to BiP, but might be expected to elute both specifically and nonspecifically bound bacteriophages. Following the first round of panning, ~30,000 of the original 1×10^{12} bacteriophages were recovered by elution with ATP, while ~4×10^7 bacteriophages were recovered after acidic elution. After amplification, aliquots of the eluted bacteriophage populations were subjected to further rounds of panning.

A nitrocellulose filter assay was used to test the ability of the eluted bacteriophages to bind to BiP (Figure 3). No BiP-binding bacteriophages were detected among 588 clones from the starting library (Figure 3a). This and additional assays indicated that the incidence of BiP-binding sequences displayed by the library is no more than 1 in 1000. Similarly, none of the small number of bacteriophage clones obtained by ATP elution after panning in the absence of BiP showed any affinity for biotinylated BiP (Figure 3b). However, after only one round of panning in

the presence of BiP, ~90% of the clones eluted with ATP reacted strongly in the nitrocellulose filter assay (Figure 3c). This percentage ranged from 70% to 90% in different experiments. When the bacteriophages were eluted at acidic pH, only ~0.5% of the clones reacted with BiP after the first round of panning (Figure 3d), and this percentage increased to only ~20% after a second round and to ~35%–40% after three rounds of panning (data not shown). These data demonstrate that acid is a much less specific eluant than ATP, so more rounds of panning were required to enrich significantly the population of BiP-binding bacteriophages. A further assay based on immunoblotting of bacteriophage proteins separated by SDS–polyacrylamide gel electrophoresis showed that BiP binds to a protein in the selected bacteriophages whose electrophoretic mobility agrees with that expected for the 418-amino acid pIII fusion protein (data not shown). A few clones (indicated in Figure 1) showed particularly strong reactivity in both assays.

Analysis of the Sequences of Octapeptides Displayed by BiP-Binding Bacteriophages

The sequences of octapeptides displayed by 114 BiP-binding bacteriophages obtained by panning and ATP elution were determined by DNA sequence analysis of the corresponding region of the bacteriophage genome (see Figure 1). Of these, 34 were chosen from the BiP-binding bacteriophages analyzed by the nitrocellulose filter assay after the first round of panning (ATP-1 clones), while 80 were picked after the second round of panning (ATP-2 clones). An additional 22 sequences were obtained for peptides displayed by BiP-binding bacteriophages selected from among the population recovered after three rounds of panning and acid elution (acid-3 clones).

The peptide sequences from the 114 ATP-1 and ATP-2 clones were first considered as a single population and compared with those from the 114 clones picked randomly from the starting library. Comparison of the overall amino acid composition of these two populations of octapeptides revealed that Trp, phenylalanine (Phe), and to a lesser extent, Leu were particularly enriched, while serine (Ser), alanine (Ala), Glu, and Lys were significantly depleted (see Figure 2B). A similar but not identical pattern of enrichment or exclusion was seen in the analysis of the sequences (see Figure 1) of 22 octapeptides displayed by bacteriophages obtained by three rounds of panning and acid elution. Serendipitously, the five residue spacer linking the variable octapeptides to the mature pIII protein (see Experimental Procedures) contains 4 out of 5 significantly excluded residues (Ala–Ser–Gly–Ser–Ala) and no enriched residues. This means that the spacer residues are unlikely to contribute to the binding activity of selected bacteriophages, allowing us to look only within the variable octapeptide sequences for the presence of a binding motif.

BiP Binds Peptides Enriched in Trp, Phe, and Leu in Alternating Positions

We then compared the starting and selected libraries in a position-dependent manner. A statistical analysis performed by maximum likelihood and bootstrap resampling (Efron, 1982) revealed that the distribution of residues by position in BiP-binding peptides is significantly different from the distribution of residues by position in the peptides from the original library (hypothesis H_0; see Experimental Procedures). More precisely, it is highly unlikely ($p < 0.0002$, based on 10,000 bootstrap replications) that both sets of peptides were randomly drawn from the same common pool. In particular, the aliphatic residues ($p < 0.0002$), the large residues ($p < 0.0002$), the hydrophobic residues ($p < 0.002$), the charged residues ($p < 0.02$), and the residues Trp/Phe/Leu ($p < 0.0002$), occur more frequently in the selected library than in the random library.

Although additional statistical analysis (hypothesis H_1) showed that the distribution of individual residues or of the subsets of residues identified above did not vary significantly by position in peptides of the selected population, there was a significant correlation ($p < 0.001$) between the intensity of reaction in the nitrocellulose filter assays and the presence of a pair of Trp/Phe/Leu residues separated by a single amino acid (hypothesis H_2). In particular, a bacteriophage that reacted strongly in these assays was twice as likely to have the pair as one that did not react strongly. A visual inspection of the selected peptides showed that shifting the frame of many of the sequences by one position (see Figure 1) exposed a seven residue motif best described as Hy(W/X)HyXHyXHy, where Hy is a large hydrophobic amino acid (most frequently Trp, Leu, or Phe), W is Trp, and X is any amino acid. This core motif is of the size determined previously to fill the peptide-binding pocket of BiP (Flynn et al., 1991). In Figure 4, the number of occurrences of each amino acid at each position of the seven residue core sequences displayed by the 114 BiP-binding ATP-1 and ATP-2 bacteriophages is compared with the number present in the core sequences of the 114 peptides from the starting library. The data shows that Trp, Phe, and Leu are highly enriched in the selected peptides, particularly at positions 2, 4, 6, and 8 (when the heptapeptide core is defined as positions 2–8). Two other hydrophobic residues, methionine (Met), and isoleucine (Ile), are also enriched but at subsets of these positions. The majority of the BiP-binding peptides contain at least two of these preferred residues at the even-numbered positions. Separate analysis of sequences from bacteriophages from the ATP-1 and ATP-2 populations showed that repeated panning leads to an increase both in the overall percentage of peptides containing two or more preferred residues in the alternating positions and in the numbers of peptides containing three or more preferred residues in these positions (see Figure 1). When only two preferred residues are present in the peptides, no bias is apparent in their relative placement; i.e., they are approximately equally distributed between the various adjacent positionings (e.g., Figure 1, positions 2 and 4, 4 and 6, 6 and 8) and the various distant positionings (e.g., positions 2 and 6, 2 and 8, 4 and 8). Similarly, peptides that contain three preferred residues in the even-numbered positions display no particular positional pattern. Taken together, these data suggest that the peptide-binding site on BiP contains four pockets that can accomodate large hydrophobic/aromatic residues and that occupancy of any

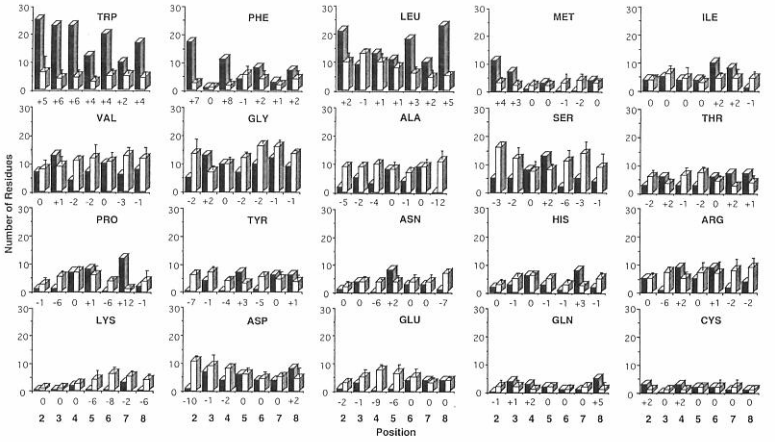

Figure 4. Comparison of the Abundance of Amino Acid Residues at Particular Positions in BiP-Binding and Nonbinding Octapeptides

The number of occurrences of each of the 20 amino acids at each position of the seven residue core sequences (defined as positions 2–8; see Figure 1) in peptides displayed by the 114 BiP-binding ATP-1 and ATP-2 bacteriophages (closed columns) is compared with the number present in core sequences of the 114 peptides from the ON141 starting library (open columns). As described in the legend to Figure 1, many of the sequences of the octapeptides from BiP-binding bacteriophages had been shifted by one amino acid position to provide the optimal alignment that reveals the heptameric BiP-binding motif in positions 2–8. Since there was no experimental basis on which to choose an optimal alignment of the heptameric core sequences of unselected peptides, the 114 octapeptides from the ON141 starting library were arranged in six possible alignments (no shifting, core heptapeptide equals positions 1–7 [renumbered 2–8]; no shifting, core heptapeptide equals positions 2–8; shift every odd-numbered peptide by one position; shift every even-numbered peptide by one position; shift peptides 1–57 by one position; shift peptides 58–114 by one position), and the number of occurrences of each of the 20 amino acids at each position of the seven residue core sequences (defined as positions 2–8) was counted for each alignment. The average of the six determinations is shown in the histogram, and the range in which the values lie is shown by the error bar. Scores were assigned for each of the 20 residues at each of the positions on the basis of the fold difference in abundance in BiP-binding peptides and in unselected peptides. These scores, which are shown below each pair of columns in the histograms, were then used to compute overall scores for peptides (see Figures 1 and 6) using the BiP Score program described in Experimental Procedures.

two of the pockets by preferred residues is sufficient to promote a stable interaction between the peptide and the BiP molecule.

Although the presence of hydrophobic residues in alternating positions provides the dominant binding motif, other residues clearly make important contributions. Glutamine (Gln) is enriched at positions 4 and 8, and several other amino acids are enriched to varying extents at the intervening odd-numbered positions, including Met, Gly, and threonine (Thr) at position 3; asparagine (Asn), Ser, and tyrosine (Tyr) at position 5; and histidine (His), Ile, proline (Pro), and Thr at position 7 (Figure 4). In most of these cases, the enrichment is relatively modest (2- to 3-fold). However, Pro at position 7 displays the greatest degree of enhancement (12-fold) observed for any amino acid at any position. Close examination of sequences containing Pro at this position reveals that it is frequently located between two amino acids with bulky side chains (particularly two Trp residues), suggesting that its role may be to prevent steric interference or stacking interactions between the two aromatic residues by introducing a kink in the peptide backbone.

Positive selection to increase the frequencies of particular residues is not the only force operating to determine the optimal sequences of BiP-binding peptides since some residues display unexpectedly low frequencies at one or more positions. For example, Ala is decreased 4- to 12-fold at positions 2, 4, and 8 (Figure 4), Asn is decreased 6-fold and 7-fold in positions 4 and 8, and Ser and Pro are decreased 6-fold in position 6. Interestingly, the two negatively charged residues, Asp and Glu, are significantly excluded from more N-terminal positions, while positively charged Lys is excluded from positions 5–8. Negative selection is relatively rare in the odd-numbered positions, although Pro and arginine (Arg) are significantly excluded from position 3, as are Lys and Glu from position 5.

A Scoring System to Predict BiP-Binding Sequences

To codify the preferences discussed above and to develop a scoring system that could be used to predict BiP-binding sites in synthetic peptides and naturally occurring polypeptides, we assigned a score to each of the possible 20 amino acids at each position of the seven residue core sequence. These scores, which range from +12 to −12 as shown below the histogram columns in Figure 4, are derived from the fold difference in the overall abundance of each residue in the peptides displayed by the selected and nonselected bacteriophage populations. We then used the computer program described in Experimental Procedures to calculate the overall score for each of the two seven residue sequences present in each octapeptide. The larger of the two scores calculated for each peptide is reported in Figure 1, and histograms describing the distribution of these scores in the two populations are shown in Figure 5A. The scores obtained for the 114 peptides from BiP-binding bacteriophages range from −10 to +38, with only 8 peptides having scores below 0. The scores for the 114 peptides from unselected bacteriophages, which showed no activity in BiP binding assays (see Figure 3a), range from −22 to +9, with the majority being 0 or negative. When the populations of peptides from the ATP-1 and ATP-2 pannings (see Figure 1) were analyzed separately, there was a significant shift to higher scores following the second panning. BiP-binding peptides from acid-eluted bacteriophages displayed scores in the range from −10 to +21 (see Figure 1). Despite the partial overlap in scores between the populations of BiP-

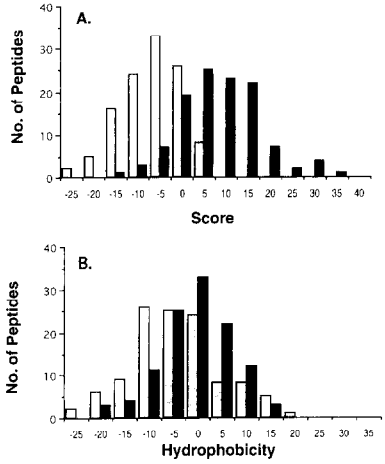

Figure 5. Distribution of BiP Scores and Hydrophobicity Scores Computed for Peptides Displayed by ON141 Bacteriophages

The distribution of the maximum BiP scores calculated for each peptide as described in the legend to Figure 1 (A) and the distribution of overall hydrophobicity scores calculated for each peptide using the hydropathy scale of Kyte and Doolittle (1982) (B) are shown for the 114 peptides displayed by bacteriophages from the original ON141 library (open columns) and the 114 peptides displayed by ON141 ATP-1 and ATP-2 bacteriophages (closed columns).

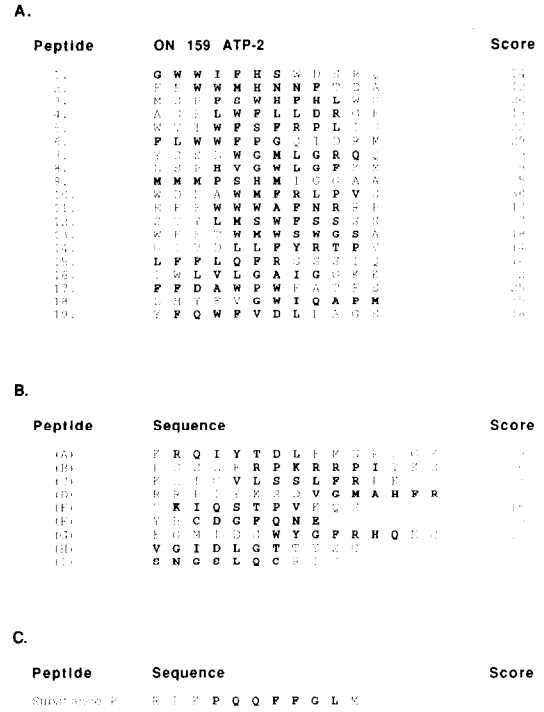

Figure 6. Amino Acid Sequences and BiP Scores of Dodecapeptides Displayed by BiP-Binding ON159 Bacteriophages and of Synthetic Oligopeptides of Known BiP-Binding Capacity

The BiP Score program described in Experimental Procedures was used to calculate the overall score for each of the overlapping heptapeptide sequences contained within (A) 19 dodecapeptides displayed by ON159 bacteriophages obtained by ATP elution after two rounds of affinity panning in the presence of BiP (ATP-2 clones).

(B) Nine synthetic peptides (denoted peptides A–I), whose capacity to interact with BiP had previously measured by Flynn et al. (1989). Of the nine peptides, which correspond to sequences positioned near the N- or C-termini of authentic viral or cellular proteins, only peptide F did not display any ATPase stimulatory activity.

(C) The 11 residue substance P neuropeptide (Oblas et al., 1990).

For each peptide, the heptapeptide sequence shown in bold typeface gives the highest score using the BiP Score program (this score is shown to the right of each peptide).

binding and nonbinding peptides, inspection of the histogram shown in Figure 5A suggests that it should be possible, using this scoring system, to identify potential BiP-binding sequences in synthetic peptides and naturally occurring proteins. Heptapeptides having a score of >10 would have an extremely high probability of binding to BiP, while peptides with scores between +6 and +10 would have odds of 3 to 1 of having binding activity. Scores from 0 to +5 would have little predictive value, but peptides with scores below 0 would almost always lack the capacity to bind to BiP.

Finally, Figure 5B shows that although the BiP-binding peptides are significantly enriched in the hydrophobic amino acids Trp, Leu, and Phe, the overall hydrophobicity of the octapeptides, calculated using the hydropathy scale of Kyte and Doolittle (1982), exhibits a nearly overlapping distribution for the populations of peptides displayed by selected and nonselected bacteriophages. Hydrophobicity per se is therefore not a useful predictor of the BiP binding capacity of the peptides. This is consistent with the fact that we did not observe significant enrichment of Ile, valine (Val), or Ala in peptides displayed by the selected bacteriophages, as would have been expected if the affinity of BiP for a peptide were simply determined by its hydrophobic character. Similarly, the Trp content of the peptides did not provide a useful predictor of their ability to bind to BiP (data not shown).

The Motif for BiP Binding Is Also Present in Dodecapeptides Displayed by Selected ON159 Bacteriophages and in Peptides of Known BiP Binding Capacity

To validate our scoring system, we needed to analyze a set of BiP-binding sequences that were not part of the data base used to generate the scoring matrix. We therefore panned a second library (denoted ON159) consisting of 5×10^8 independent recombinant bacteriophages displaying random dodecapeptides. As had been observed for the ON141 octapeptide library, the starting population of bacteriophages displayed a very low proportion of BiP-binding sequences. However, there was significant enrichment of BiP-binding bacteriophages following one or two rounds of panning with biotinylated BiP using ATP elution, while panning followed by acidic elution resulted in a much lesser degree of enrichment (data not shown). Sequences were determined for peptides displayed by 19 BiP-binding bacteriophages obtained by ATP elution through two rounds of panning (Figure 6A) and 34 BiP-binding bacteriophages selected by acidic elution through three rounds of panning (data not shown). Every ATP-2 peptide contains at least one heptapeptide (shown in bold in Figure 6A) that

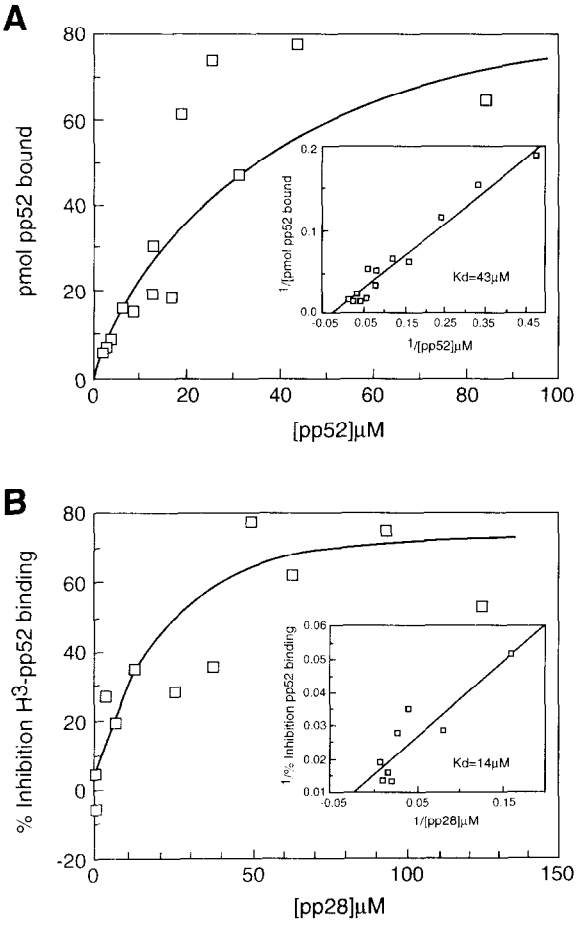

Figure 7. Analysis of the Binding to BiP of Synthetic Peptides Corresponding to Sequences Displayed by ON141 Bacteriophages

The specific binding of ^{3}H-pp52 to murine BiP (64 pmol), measured as described in Experimental Procedures, is shown in (A). The average of the K_D values determined in three different experiments was 33 ± 10 μM. At saturation, the stoichiometry was found to be 1.1 ± 0.2 molecules of pp52 per BiP monomer. The apparent binding affinity of peptide pp28, measured by competing the binding to BiP of ^{3}H-pp52 as described in Experimental Procedures, is shown in (B). The average of the K_D values determined in two different experiments was 17 ± 3 μM.

displays the Hy(W/X)HyXHyXHy motif with two or more of the Hy positions occupied by preferred residues. There is no preferred placement of the motif within the dodecapeptides (Figure 6A), indicating that as expected for a binding site designed to recognize sequences within an extended polypeptide chain, the motif need not be localized at the N-terminus of the protein. In validation of the scoring system, all the peptides score in the range expected for BiP-binding sequences. The ATP-2 peptides have maximal scores between +2 and +30 (Figure 6A). Of the 34 acid-3 peptides, 32 contain heptapeptides having scores of 0 or greater, with the highest score being +34.

We have also computed the scores for peptides that we or others have previously shown to bind to BiP or stimulate its ATPase activity. Figure 6B shows the scores obtained for the set of synthetic peptides whose capacity to interact with BiP was measured by Flynn et al. (1989). Of the nine peptides that correspond to sequences positioned near the N- or C-termini of authentic viral or cellular proteins, eight stimulated the ATPase activity of bovine BiP. All eight contain heptapeptides with scores (0–15; see Figure 6B) that lie in the range expected for BiP-binding sequences (see Figure 5A). That these scores lie in the lower half of the range is not surprising, since the peptides were chosen "off the shelf," rather than being selected for their BiP binding ability. Peptide F, which did not display any ATPase stimulatory activity, had a score of 0, which also lies in the normal range for peptides that do not bind to BiP. Figure 6C shows that the neuropeptide substance P, which binds BiP with an affinity in the micromolar range (Oblas et al., 1990), contains two heptapeptide sequences with scores of +7. Finally, BiP-binding synthetic peptides corresponding to N-terminal sequences of influenza hemagglutinin and the tumor suppressor protein, p53, also contain high scoring heptapeptide sequences (S. B.-E. and A. Fourie, unpublished data).

Synthetic Peptides with Sequences Corresponding to Those on Selected Bacteriophages Bind BiP and Elicit Its ATPase Activity

We have measured the ability of synthetic octapeptides with sequences corresponding to those displayed by six

Table 1. Equilibrium Constants for the Binding to BiP of Synthetic Peptides

Peptide	Sequence	Intensity	Score	K_d (μM)	K_M (μM) (ATPase Stimulation)
pp12	LFWPFEWI	+	+15	ND	50 ± 5
pp28	HWDFAWPW	+++	+32	17 ± 3	10 ± 2
pp37	FTYGSRWL	+	+7	ND	60 ± 5
pp38	FWGLWPWE	+++	+34	ND	15 ± 5
pp48	DGVGSFIG	+	+4	ND	40 ± 10
pp52	YVDRFIGW	+	+5	33 ± 10	ND
np53	AGEYYAAL	−	0	>1000	>1000

The numbers of the peptides are those assigned in Figure 1 to ON141 ATP-2 bacteriophages that display octapeptides sequences corresponding to those of the synthetic peptides. The intensity of the alkaline phosphatase staining observed using the nitrocellulose filter assays is a measure of the binding of BiP to these bacteriophages, and the scores shown are the larger of the two scores calculated for each peptide using the BiP Score program as described in the legend to Figure 1. K_D values were measured using the direct binding assay or the competition assay described in Experimental Procedures, and the values shown are each the average of three experiments. K_M values correspond to the concentration of peptide required for half-maximal stimulation of the ATPase activity of bovine BiP, measured as described in Experimental Procedures. The values shown are the averages of at least two experiments. ND, not determined.

BiP-binding bacteriophages and one nonbinding bacteriophage to bind to BiP (using direct binding or a competition assay) or to stimulate its ATPase activity. The sequences and scores of these peptides and the results of the assays are listed in Table 1. A K_D of 33 ± 10 μM was measured for pp52 in a direct binding assay, and a K_D of 17 ± 3 μM was measured for pp28 by competition of the binding to BiP of ^{3}H-pp52 (Figure 7; Table 1). The negative control peptide, np53, did not compete for the binding of ^{3}H-pp52 even at a concentration of 1 mM. We have recently reported that the peptide-mediated stimulation of the ATPase activity of BiP reflects the conversion of oligomeric forms of the protein into active monomers (Blond-Elguindi et al., 1993) and therefore provides a measure of peptide binding affinity. We have already shown that pp52 induces the dissociation of oligomers of bovine BiP (Blond-Elguindi et al., 1993). Consistent with its inability to bind to BiP, np53 did not induce the dissociation of BiP oligomers (data not shown) and did not stimulate the ATPase activity of the protein (Table 1). Peptides pp12, pp28, pp37, pp38, and pp48 all stimulate the ATPase activity of bovine BiP, with half-maximal stimulation occurring at peptide concentrations in the range of 10 to 60 μM (Table 1). The two peptides that apparently have the highest apparent affinities for BiP (pp28 and pp38) are the ones that conform very well to the Hy(W/X)HyXHyXHy motif, have the highest scores (+32 and +34), and were included in the class of strong (triple plus) positives in the original screening assays (see Figure 1).

Discussion

The molecular chaperone BiP recognizes (poly)peptides that contain a heptameric motif best described as Hy(W/X)HyXHyXHy. Our data suggest that the peptide-binding site on BiP contains four pockets that can accomodate the side chains of these large hydrophobic and aromatic amino acids (Figure 8) and that occupancy of any two of the pockets by preferred residues is sufficient to promote a stable interaction between the peptide and the BiP molecule. The motif has a high degree of redundancy, consistent with the capacity of BiP to recognize a wide variety of nascent polypeptides that share no obvious sequence similarity, and it contains a preponderance of residues whose side chains would normally be buried in the interior of a folded protein, consistent with the ability of BiP to discriminate accurately between properly folded and unfolded structures. The high abundance of Trp residues in the BiP-binding octa- and dodecapeptides identified in this study is noteworthy given the particularly low abundance of this amino acid in most natural proteins. Its predominance in our analysis may reflect the fact that these bacteriophage-displayed peptides were selected from a population whose amino acid composition had not been constrained by the necessity to fold into a compact three-dimensional structure. It is possible that a heptapeptide containing four Trp residues in the Hy positions of the motif would, if soluble, represent an ideal high affinity BiP-binding sequence. However, within a naturally occurring polypeptide, such a sequence might have deleterious effects if, in vivo, BiP and the folding polypeptide need to interact with relatively low affinity to facilitate their transient association. Synthetic octapeptides containing the motif bind to BiP with apparent affinities in the range 10–60 μM, corresponding to the highest values reported so far for BiP–peptide complexes (Flynn et al., 1989). We do not know whether these in vitro measurements accurately reflect the in vivo situation in the ER, where interactions between BiP and its polypeptide substrates may be modulated by the physicochemical environment and by the presence of cochaperones, such as homologs of DnaJ (Langer et al., 1992; Sanders et al., 1992).

Our finding that the presence of hydrophobic amino acids is an important, although not sufficient, feature of BiP-binding peptides is consistent with the observation of Flynn et al. (1991) that a population of water-soluble heptameric peptides bound to BiP were enriched in amino acids with aliphatic side chains, particularly at positions 3–6. However, Flynn et al. were unable to observe the significant enrichment of the aromatic residues Trp (a residue that could not be analyzed in their study) or Phe (whose abundance is particularly increased in the first binding position [Figure 4] for which they could not obtain data). Furthermore, they did not observe the alternating pattern of hydrophobic residues, perhaps because they were unable to define the individual sequences of their BiP-binding peptides and therefore had no opportunity to perform the alignment necessary to reveal the motif.

An alternating pattern of Hy residues is compatible with peptides being bound to BiP in an extended conformation, with the bulky hydrophobic side chains lying on one side of the strand and pointing into a binding cleft on the BiP molecule (see Figure 8). This model is consistent with nuclear magnetic resonance studies of the binding of peptides to BiP (S. B.-E., S. Landry, and L. Gierasch, unpublished data) and to its homolog in E. coli, DnaK (Landry

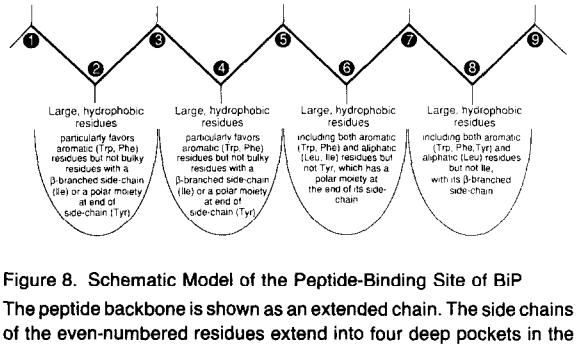

Figure 8. Schematic Model of the Peptide-Binding Site of BiP

The peptide backbone is shown as an extended chain. The side chains of the even-numbered residues extend into four deep pockets in the peptide-binding site that have similar overall preferences for large hydrophobic or aromatic side chains. However, there is some variation between the pockets in the degree to which individual residues are preferred or excluded. Although not shown, there may in addition be one or two minor pockets on the other side of the cleft or residues positioned at the top of the cleft whose side chains can interact with those of the odd-numbered residues of the peptide. Thus, the data shown in Figure 4 indicate that particular amino acids at peptide positions 3 (Trp and Met) and 5 (Trp and the polar residues Tyr, Ser, and Asn) may make favorable contacts with the BiP side chains. On the other hand, charged residues may make particularly unfavorable contacts at these positions.

et al., 1992), which indicate that peptides are bound in an extended conformation. The peptide-binding site on BiP is likely to contain deep pockets in which small hydrophobic residues (Ala or Val) cannot establish stable interactions. The four major pockets in the binding cleft have similar overall preferences for large hydrophobic or aromatic side chains, although there is some variation between them in the degree to which individual residues are preferred or excluded (see Figure 8). There may be, in addition, one or two minor pockets on the other side of the cleft or residues positioned at the top of the cleft, whose side chains can interact with those of the odd-numbered residues of the peptide.

The arrangement shown in Figure 8 is reminiscent of that described for the binding of peptides to major histocompatibility antigens (Fremont et al., 1992; Madden et al., 1992; Brown et al., 1993), and, indeed, two groups have proposed that the three-dimensional structure of the C-terminal peptide-binding domains of hsp70 proteins resemble that of the peptide-binding $\alpha 1\alpha 2$ superdomain of the human major histocompatibility complex class I antigen, histocompatibility leukocyte antigen (Rippmann et al., 1991; Flajnik et al., 1991). Peptides (usually octamers or nonamers) bind to class I molecules in an extended conformation in a binding groove that contains six pockets or subsites which vary in their relative depth and size (Saper et al., 1991; Matsumura et al., 1992). Two deep pockets accomodate the N- and C-termini of the peptide, and the high affinity (nanomolar range) of peptide binding to class I is largely due to extensive hydrogen bonding between these termini (and other main chain atoms in the peptide) and side chain atoms of the protein. A third deep pocket found in different positions in different alleles plays the major role in determining allele-specific peptide binding by providing chemical and structural complementarity for a particular anchor residue (Rammensee et al., 1993). The other shallower pockets accomodate the side chains of a more extensive subset of amino acids and play an apparently minor role in peptide binding specificity (Saper et al., 1991; Matsumura et al., 1992). Class II molecules bind longer peptides (12–24 residues) in an extended conformation stabilized by hydrogen bonding between main chain peptide atoms and some conserved residues in the peptide-binding cleft. The cleft contains one deep pocket lined with nonpolar residues that is likely to accommodate the dominant aromatic anchor residue of the bound peptide, as well as three other pockets likely to bind the side chains of a secondary anchor residue and two allele-specific residues positioned 3, 5, and 6 or 8 amino acids further along the peptide (Brown et al., 1993; Hammer et al., 1993). Even if the overall structures of the peptide-binding domains of major histocompatibility complex and hsp70 molecules are similar, our results suggest that the details of the binding interactions will vary. First, unlike class I, BiP and other hsp70 molecules bind sequences that are embedded in long polypeptide chains so that free N- and C-termini are not available for hydrogen bonding. In this respect, hsp70 proteins more closely resemble class II molecules, which bind longer peptides that project out of both ends of the binding cleft (Brown et al., 1993). Second, the fact that the BiP-binding motif involves hydrophobic and aromatic residues (but not Tyr) suggests that hydrogen bonds to atoms in the peptide side chains do not contribute significantly to the interaction between hsp70 molecules and their (poly)peptide ligands. Such bonds require specific geometries of interaction, which would be incompatible with recognition by hsp70 proteins of a very wide variety of sequences. Third, peptides that bind BiP do not contain a single dominant anchor residue. Rather, the more generalized requirement for the binding of large hydrophobic residues in any two or more of the pockets facilitates recognition of an even greater variety of peptides than is observed for major histocompatibility complex molecules, while maintaining the ability to discriminate between sequences likely to be exposed by properly folded and unfolded or unassembled structures.

Biochemical evidence from studies in yeast indicates that BiP interacts with nascent polypeptide chains during their translocation into the lumen of the ER (Sanders et al., 1992). As discussed above, hemagglutinin and other secretory proteins contain potential BiP-binding sites located close to the mature N-terminus of the polypeptide chain. In addition, analysis using the BiP Score program of a collection of 85 signal sequences compiled by von Heijne (1983) indicates that they all contain potential BiP-binding sequences, the great majority having scores between 10 and 31. Whether or not a direct interaction between BiP and the signal sequence on a nascent polypeptide plays any role in facilitating its translocation across the ER membrane or in presentation of the polypeptide for signal cleavage can be tested by mutagenesis.

Two alternative models for the role played by BiP during protein folding and assembly in the ER are distinguished by the degree to which the pathway of folding of a newly synthesized polypeptide is modulated by binding to the chaperone. The first involves a passive involvement of BiP in which its binding merely prevents off-pathway interactions that result in misfolding and aggregation. The second model incorporates the additional feature that secretory polypeptides may have evolved to contain recognition motifs with a hierarchy of affinities that directs their order of release from the chaperone. They would thus take advantage of BiP (which would possess no informational content regarding the final structure of the protein) to modulate the order in which different regions or domains of the polypeptide become available for folding. To understand how BiP distinguishes between folded and unfolded structures and to discriminate between the models described above, we need to know where and how often high (or low) affinity binding sites occur in natural proteins. We are currently testing our model on a protein of known three-dimensional structure: the hemagglutinin of influenza virus. The BiP Score program predicts that this 547 amino acid polypeptide contains approximately 15 potential BiP-binding sites, which in the properly folded protein are usually located either in the interior of a folded domain or at the interface between subunits. Measurement of the relative affinities for BiP of synthetic peptides corresponding to the potential binding sequences, coupled with site-directed mutagenesis of the protein to strengthen or weaken the binding mo-

tifs, should provide insight into their role during the folding and assembly of the hemagglutinin molecule.

Experimental Procedures

Purification and Biotinylation of BiP

Murine BiP was expressed in E. coli, purified, and assayed as described (Blond-Elguindi et al., 1993). The purified protein (1 mg/ml) was dialyzed against 0.1M sodium bicarbonate (pH 8.4) for 2 hr at room temperature. The protein was biotinylated using sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS–LC–biotin) according to the instructions of the manufacturer (Pierce) and then dialyzed at 4°C against phosphate-buffered saline (PBS) containing 0.1% sodium azide. The extent of biotinylation of BiP was estimated using the 2-(4′-hydroxyazobenzene) benzoic acid test (HABA test, Pierce) to be 1–1.5 molecules of biotin per molecule of BiP. The basal ATPase activity of the biotinylated BiP was 90%–98% of that of the unmodified protein. Furthermore, biotinylated BiP binds to synthetic peptides as measured by peptide-dependent stimulation of its ATPase activity (data not shown). Bovine BiP used for ATPase assays was a gift of Dr. G. Flynn (Oregon Health Sciences Center).

Construction of the Random Peptide Expression Library

Libraries of bacteriophages expressing random octapeptides or dodecapeptides were constructed by a modification of the method described previously (Cwirla et al., 1990). To construct the ON141 library, a collection of oligonucleotides encoding octapeptides fused to the spacer sequence Ala–Ser–Gly–Ser–Ala was synthesized with the sequence 5′-CTC TCA CTC CNN KNN KNN KNN KNN KNN KNN KGC AAG TGG CTC TGC TAC TGT TGA AAG TTG T-3′ in which N is A, C, G, or T (equimolar) and K is G or T (equimolar). Two half-site oligonucleotides, 5′-GGA GTG AGA GTA GA-3′ (ON-28) and 5′-CTT TCA ACA GT-3′ (ON-29), complementary to the 5′ and 3′ ends of ON141 and ON159 oligonucleotides, were also synthesized. Annealing of the oligonucleotides, their insertion into the BstXI site of fd phage affinity vector 1 (fAFF1), transformation of the ligated DNAs by electroporation into E. coli MC1061, and isolation of recombinant bacteriophage particles were performed as described previously (Cwirla et al., 1990). This process yielded 1.4×10^9 independent recombinants, of which 80% produced infective bacteriophage particles. Amplification of the primary library produced approximatively 1×10^{14} transducing units when assayed on E. coli K91 cells (Parmley and Smith, 1988). Essentially the same procedure was used to construct the ON159 library except that the collection of oligonucleotides encoding dodecapeptides fused to a Gly–Gly spacer had the sequence 5′-CTC TCA CTC CNN KNN KNN KNN KNN KNN KNN KNN KNN KNN KGG CGG CAC TGT TGA AAG TTG T-3′. This process yielded 5×10^8 independent recombinants.

Selection of BiP-Binding Bacteriophages by Affinity Panning

Bacteriophages displaying peptides recognized by BiP were selected by a modification of the affinity panning method (Parmley and Smith, 1988). Eight wells of a microtitration plate were coated with streptavidin by incubation with 50 μl/well of a solution of the protein (10 μg/ml in PBS) for 1 hr at 37°C. The wells were then washed three times with PBS containing 0.05% Tween 20 (PBS–Tween) and then blocked by incubation for 1 hr at 37°C with 1% bovine serum albumin in PBS. Biotinylated BiP (50 μl/well of a 20 μg/ml solution in PBS) was then added to six of the wells, while the remaining two wells received PBS only. Following incubation for 2 hr at 4°C, the wells were washed three times with PBS–Tween. ON141 or ON159 bacteriophages (1000 library equivalents) were precipitated by the addition of 1/10 volume of 1 M acetic acid, incubated on ice for 10 min, then collected by centrifugation (10,000 × g for 20 min), and resuspended in 800 μl of buffer A (20 mM HEPES [pH 7.0], containing 20 mM potassium chloride, 2 mM magnesium chloride and 10 mM ammonium sulfate. The resuspended bacteriophages (100 μl) were added to each of the eight microtiter wells, while a small aliquot was retained for titration of the bacteriophages. Following incubation for 2 hr at 4°C and six washes with cold PBS, the bound bacteriophages were eluted either by acidic treatment as described (Cwirla et al., 1990) or by incubation at room temperature with ATP (3 mM in buffer A) for 5 hr or overnight. The eluates from wells with BiP and wells without BiP were pooled separately, and aliquots were kept for titration of the eluted bacteriophages. The remaining bacteriophages were immediately amplified by infection of an exponentially growing culture of E. coli K91 cells prepared as follows: a 50 ml culture of K91 cells was grown in LB until OD_{600} was 0.5. The cells were harvested by centrifugation, resuspended in 5 ml of LB, and kept on ice until needed. Typically for amplification, 200 μl of resuspended K91 cells was mixed with 200 μl of eluted bacteriophages, incubated for 20 min at 37°C, and spread on a large plate of LB agar containing 20 μg of tetracycline per milliliter. After incubation overnight at 37°C, the cells were harvested by adding 10 ml of LB per plate and gentle scraping of the bacterial cells. The cells were centrifuged, and the amplified bacteriophages in the supernatant were collected by precipitation with 0.2 vol of 20% polyethylene glycol containing 2.5 M sodium chloride. After incubation for 1 hr at 4°C, the bacteriophages were pelleted and resuspended in 800 μl of PBS. The amplified bacteriophages were then ready for subsequent rounds of panning. For titration, 10 μl of an appropriate dilution of the bacteriophages was added to 50 μl of K91 cells. Following incubation for 20 min at 37°C, 100 μl of LB was added, and the cells were spread on LB plates containing 20 μg of tetracycline per milliliter.

Screening for BiP-Binding Bacteriophages

Clones of tetracycline-resistant cells from plates containing 50–200 colonies were transferred to nitrocellulose filters. The bacteria were washed from the filters by squirting with PBS containing 0.05% Tween 20 and 1% bovine serum albumin, and the filters were then incubated for 30 min in the same buffer before washing three times with PBS–Tween. Following incubation for 1 hr in 5 ml of biotinylated BiP (0.1–2 μg/ml in PBS–Tween), the filters were again washed three times in PBS–Tween. The positions of clones that had secreted BiP-binding bacteriophages were then located by one of two methods: the filters were incubated with 5 ml of alkaline phosphatase-conjugated streptavidin (1/10,000 dilution in PBS–Tween; Pierce) for 1 hr at room temperature before extensive washing with PBS–Tween, or the filters were incubated for 1 hr in 5 ml of an anti-biotin antibody (1/50,000 dilution in PBS–Tween; Pierce), washed, and incubated with a rabbit anti-goat immunoglobulin antibody conjugated to alkaline phosphatase (1/5,000 dilution in PBS–Tween; Pierce). In each case, alkaline phosphatase activity was revealed using a mixture of nitroblue tetrazolium/and 5-bromo-4-chloro-3-indolylphosphate toluidinium (Bethesda Research Laboratories) as substrate.

Determination of the Sequence of Bacteriophage-Displayed Peptides

Single-stranded bacteriophage DNAs were purified and sequenced by the dideoxy chain termination method (Sanger et al., 1977) using as primer an oligonucleotide (5′-CGA TCT AAA GTT TTG TCG TCT-3′) complementary to a sequence 40 nt 3′ of the second BstXI site in fAFF1 (Cwirla et al., 1990). Sequencing reactions were carried out using the Sequenase kit as advised by the manufacturer (U. S. Biochemical).

Statistical Analysis

The hypotheses tested were as follows: H_0, the peptides drawn from the random library and those drawn from the selected library are actually drawn from a common pool by a common procedure; H_1, the distribution of residues in the selected peptides is independent of position; H_2, there is no relationship between the occurrence of at least one pair of Trp/Phe/Leu residues separated by a single amino acid in the peptide and the intensity of alkaline phosphatase staining in the nitrocellulose filter assays. The first two hypotheses were applied to the distribution of individual residues and to groups of residues and tested with a likelihood ratio statistic and Monte Carlo bootstrapping (Efron, 1982). The groups of residues used were as follows: aliphatic/nonaliphatic; aromatic/nonaromatic; large/small or tiny; charged/neutral or polar/nonpolar. Amino acid membership of the various groupings was as defined previously (Taylor, 1986). The third hypothesis was tested using Fischer's exact test.

A Computer Program to Score BiP-Binding Peptides

The BiP Score computer program was written to detect potential BiP-binding sites contained within protein primary sequences. For each seven residue sequence, the program computes a score:

$$S(n) = \sum_{i=n+6}^{i=n} s(i,p)$$

where $s(i,p)$ is a parameter related to the probability of finding a residue of type p (1 of the 20 amino acids) at position i in the seven residue sequence beginning at position n in the polypeptide sequence. The values of $s(i,p)$ were determined by comparison of two groups of 114 heptapeptides that differ in their ability to bind to BiP. Where $f(i,p)$ and $F(i,p)$ are the frequencies with which an amino acid of type p occurs at position i in peptides from the unselected and selected populations, respectively, then $s(i,p) = F(i,p)/f(i,p)$ when $F(i,p) > f(i,p)$ but $s(i,p) = -f(i,p)/F(i,p)$ when $F(i,p) < f(i,p)$. Values of $s(i,p)$ are stored in an ASCII file that is accessed by the scoring program.

Peptide Synthesis

Octapeptides were prepared by continous flow solid-phase synthesis and analyzed by high pressure liquid chromatography and mass spectrophotometry as described in Blond-Elguindi et al. (1993). ^{3}H-labeled peptides were prepared by incubating peptide–resin (Fmoc) or cleaved peptide with [^{3}H]acetic anhydride (500 mCi/mmol; Amersham International). The specific activity of the labeled peptides varied from 8 to 25 cpm/pmol.

Assays of Peptide Binding to BiP

For direct analysis of peptide binding, 5 μg or 20 μg of recombinant mouse BiP (64 pmol or 256 pmol) was incubated with ^{3}H-labeled peptides (0–10 nmol of pp52, 0–90 nmol of np53) in a final volume of 50 μl of 20 mM HEPES (pH 7.0) containing 20 mM sodium chloride and 3 mM magnesium chloride for 2 hr at 37°C. The peptide–BiP complexes were separated from free peptide by precipitation with a final concentration of 10% trichloroacetic acid followed by filtration on Whatman GF/C filters. The filters were washed with 50% trichloroacetic acid and 90% ethanol and dried, and the bound radioactivity was determined by scintillation counting. Background counts were determined at each peptide concentration for parallel samples that did not contain BiP and were subtracted. In competition experiments, 64 pmol of BiP (1.28 μM final concentration) and 2.15 nmol of ^{3}H-pp52 (43 μM final concentration) were incubated with 0–6 nmol of pp28 (0–124 μM final concentration). The fraction of bound peptide was determined as described above.

ATPase Assays

Assays of the ATPase activity of bovine BiP were performed as described in Blond-Elguindi et al. (1993). Peptide-mediated stimulation of this activity was assayed in the presence of synthetic peptides over a concentration range from 1 μM to 1 mM or to the limit of solubility of the peptide.

Acknowledgments

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