A Phage Display-based Method for Determination of Relative Affinities of Mutants

APPLICATION TO THE ACTIN-BINDING MOTIFS IN THYMOSIN β4 AND THE VILLIN HEADPIECE

We propose phage display combined with enzyme-linked immunosorbent assay as a tool for the systematic analysis of protein-protein interactions by investigating the binding behavior of variants to a partner protein. Via enzyme-linked immunosorbent assay we determine both the amount of fusion protein presented at the phage surface and the amount of complex formed, the ratio of which is proportional to the affinity. Hence this method enables us to calculate the relative affinities of a large number of mutants. As model systems, we investigated actin-binding motifs conserved in a number of proteins binding monomeric or filamentous actin. The hexapeptide motifs LKKTET, present in thymosin β4, and LKKEKG, present in the villin headpiece, were mutated, and the variants were analyzed. Study of the positional tolerance allows postulating that the motifs, although similar in primary structures adopt different conformations when bound to actin. In addition, our data show that the second and the fourth amino acid of the thymosin β4 motif and the first three residues of the villin headpiece motif are most important for actin binding. The latter result challenges the charged crown hypothesis for the villin headpiece filamentous actin interaction.

As a consequence of various genome-sequencing projects, novel proteins are being identified, many of which may take part in new interactions. Efforts are going on to tackle the discovery of protein-protein interactions globally (1), but there is also need for novel methods for the analysis of these interactions, preferably easy, reliable, and high throughput techniques. Several means to probe amino acids that contribute to the binding of two proteins have been developed. X-ray crystallography and NMR may yield superior information about the interface of proteins at atomic resolution (2, 3). However, both methods are intrinsically difficult (even for noncomplexed proteins) and time-consuming and require expensive and sophisticated equipment, as well as large amounts of biological material. Cross-linking of interacting proteins followed by mass spectroscopy or conventional sequence determination of covalently coupled peptide fragments has limited application (4–6). Probing the accessible surface in protein complexes by deuterium exchange has also been employed (7). But by far the two most popular methods are the use of peptide mimetics either in solution (8) or on membranes (9, 10) and especially site-directed mutagenesis (11, 12). However, in the latter powerful technique to study functions of proteins or interactions between proteins, one often faces the difficulty of choosing the position and type of amino acid exchange to be introduced. To circumvent this, one may perform a saturation mutagenesis at several positions thought to be important for the interaction. Obviously this creates a new problem, i.e. screening a large number of mutants in a systematic way.

We propose to combine saturation mutagenesis, phage display and ELISA for the systematic screening and quantification of protein-protein interactions. Phage display is traditionally used for the selection of stronger binding ligands against target molecules (13); here we employ it differently. We generate defined libraries of mutants and subsequently analyze all of the different recombinant phages using ELISA. In this ELISA we determine both the amount of fusion protein on the phage and the amount of complex formed. The ratio of both gives information about the binding strength of the different mutants. We apply this method to the presentation of thymosin β4, analyzing monomeric actin (G-actin) binding, and of the villin headpiece, probing both G-actin and filamentous actin (F-actin) binding.

We selected these actin-binding modules because for thymosin β4 the actin binding information is relatively well characterized, but its structure is poorly defined. By contrast the villin headpiece has a well resolved NMR structure, but information on those residues that interact with actin is still elusive. Thymosin β4 interacts with actin via residues in an α-helix and a conserved hexapeptide motif (LKKTET) (14–16). NMR studies of thymosin β4 reveal no unique structure for this motif; however, it is evident that the motif must become structured upon binding actin (17–19). A seemingly related sequence (LKKEKG) is present in a set of C-terminal headpiece domains (15, 16) implicated in F-actin binding (20). NMR of the villin headpiece showed that the first five amino acids of the

© 2003 by The American Society for Biochemistry and Molecular Biology, Inc.
motif are in a α-helical conformation (21, 22), and some of these are part of a charged crown suggested to be important for actin binding (20). Although both modules appear to have analogous motifs in their primary structure, they display different specificities for G- and F-actin. This suggests that local structural changes in these actin-binding units govern the recognition of the different conformational states of actin.

The definition that a combination of mutagenesis, phage display, and ELISA may be a powerful new tool to systematically investigate protein-protein interactions by evaluating the relative binding strengths of mutants. Next to mutational tolerance at a position, structural information on the bound conformation can be inferred from mutants displaying increased affinity. In addition, our results show that some of the charged crown residues in the villin headpiece, hypothesized to be important for actin interaction, are dispensable for actin binding.

**EXPERIMENTAL PROCEDURES**

**Construction of the Libraries and Rescue of the Recombinant Phages**—Enzymes and reagents for molecular cloning were purchased from New England Biolabs or Invitrogen and were used following the manufacturer’s instructions. The oligonucleotides were synthesized by Eurogentec. The phagemid vector pCANTAB5E, the detection module, and the Escherichia coli strain TG1 were from Amersham Biosciences. The helper phage M13KO7 was from Promega. The Sequenase version 2.0 kit was from U.S. Biochemical Corp., and [γ-32P]dATP was from ICN.

We used the phagemid pCANTAB5E harboring the wild type human thymosin β4 or human villin headpiece cDNA, inserted between the SfiI and NotI restriction sites, as starting material to create the libraries. We constructed this library by independently mutating each of the six codons of both hexapeptide motifs into the 63 other possible ones, using overlap extension PCR mutagenesis (23) with synthetic oligonucleotides completely degenerated at the desired positions. The obtained PCR products were SfiI- and NotI-ligated in pCANTABSE. The resulting phagemid contains under control of the lac promoter, the gene III (gIII) signal coding sequence, the thymosin β4 or the villin headpiece gene, the E-tag coding sequence, an amber codon (TAG), and the rest of the gIII coding region. The ligation was electrotransformed in TG1 cells. Phagemid DNA of individual transformants, was purified with Wizard™ miniprep kits (Promega) and sequenced. These isolated transformants were used following the manufacturer’s instructions. Various concentrations of purified actin were used following the elution procedure of F-actin (28). The ELISA was carried out as above with similar phage dilutions as used for thymosin β4. Based on the higher values, the amount of presented villin headpiece was a factor 2–5-fold higher than that of thymosin β4.

**Other Binding Assays**—The Kd values of chemically synthesized thymosin β4 variants (24) were determined as in Ref. 15 except that we used phosphate-buffered saline, the buffer also used in the ELISA. The genes coding for wild type or mutant villin headpiece were cloned from the pCANTAB5E phagemid in pET11d (Novagen), with a new stop codon inserted at the 3′-end of the villin headpiece coding sequence and expressed in E. coli MC1061 containing pSCM26 (29). The proteins, thus lacking the E-tag sequence, were purified following the protocol in Ref. 22. The Kd values for the villin headpiece mutants were determined in a sedimentation assay. We incubated 12 μM F-actin with various concentrations of the villin headpiece, or mutant, for 2 h at room temperature and sedimented the F-actin with an Airfuge (Beckman) for 15 min at 100,000 × g. The supernatant was removed, and the F-actin pellet was washed with G buffer supplemented with 100 mM KC1 and 1 mM MgCl2. Aliquots of the supernatant and pellet were analyzed using a 10% Tricine gel. After staining the gels, we used densitometric scanning to determine the amount of bound and free villin headpiece. We used these values in a Scatchard plot to calculate the Kd. The Kd for the WT-villin headpiece F-actin interaction is ~10-fold higher than previously reported using slightly different buffer conditions at 4 °C (20). The Kd of the E-tag antibody for the E-tag sequence was determined using a Bicore X. The antibody was coated to a CMS chip (Biacore, Sweden) in HEPES-buffered saline with 150 mM NaCl, 5 mM ethyl-3(3-dimethylaminopropyl)carbo
diimide hydrochloride and N-hydroxysuccinimide chemistry according to the manufacturer’s instructions. Various concentrations of puri-

*S. Rossenu, S. Leyman, and C. Ampe, unpublished results.*
Gene of interest or library of mutants

Ligation (in phagemid pCANTAB5E)

Sfi I

E. coli (TG1)

Individual clones → sequence determination

Monovalent display

ELISA

Wells coated with E-tag antibody

Wells coated with Target protein X

\( K_d [X_{tot}] + 1 = A_{0}/A \)

FIG. 1. A phage display strategy for studying protein-protein interactions. Wild type and/or mutant genes are ligated in the phagemid, here Sfi I- and Not I-restricted pCANTAB5E, between the coding sequence of the gIIIp signal(s) and the coding sequence for the E-tag followed by the remainder of the gIIIp gene. The phagemid is electro- porated in the appropriate E. coli strain. Individual clones are grown both for rescue and preparing the phagemid DNA for sequencing. In ELISA one measures, for the obtained recombinant phages, \( A_0 \) and \( A \) proportional to the total amount of fusion protein (via the E-tag) and the amount of formed complex with the target protein, respectively. The ratios \( A_0/A \) are in linear correlation with the \( K_d \) value, and \( [X_{tot}] \) is the total target protein concentration, here actin (for details see "Experimental Procedures").

### TABLE I

**Codon usage influences TIA values but not \( A_0/A \) values**

| codon | Frequency | TIA value | \( A_0/A \) value |
|-------|-----------|-----------|-----------------|
| AGG (Arg) | 0.5 | \( 1.16 \times 10^{11} \) | 32.8 |
| CGG (Arg) | 0.5 | \( 1.11 \times 10^{11} \) | 34.1 |
| CGT (Arg) | 1 | \( 1.25 \times 10^{11} \) | 32.4 |
| AAG (Lys, WT) | 26 | \( 2.62 \times 10^{7} \) | 33.6 |

**RESULTS**

Evaluating the contribution of amino acids participating in the interaction of two proteins is often difficult and time-consuming, especially when three-dimensional structures of the individual proteins or of the complex are unknown. In the post-genome era, in which researchers employ bioinformatic
to predict new potential interactions, there is a need for relatively fast and easy ways to investigate the binding of two proteins to confirm these predictions. In a previous study, we already explored the possibility of using a combination of three existing methods: PCR, phage display, and ELISA to study protein-protein interactions (24). In the present work we improved this method and applied it to the interaction of thymosin \( \beta 4 \) with G-actin and of villin headpiece with G- and F-actin using libraries of mutants.

**Creation and Completeness of the Libraries of Thymosin \( \beta 4 \) and Villin Headpiece**—Using double cycle PCR saturation mutagenesis reactions (23) on the thymosin \( \beta 4 \) and the villin headpiece gene, inserted in the pCANTAB5E phagemid, we created libraries for each of the six positions of the hexapeptide motifs of both actin-binding modules. In the case of thymosin \( \beta 4 \), the motif is known to be important for the interaction with actin (14, 15); for the villin headpiece this has been proposed (22, 30). For each of the 12 libraries we obtained at least 2–5 × 10^6 transformants/\( \mu \)g DNA in E. coli, far in excess over the expected 64 variants for each position in the motif. From each of the six thymosin \( \beta 4 \) libraries, we isolated on average 70 clones and sequenced them to identify the created mutation. We recovered 103 of the 114 possible mutants at the amino acid level. From the results presented below for thymosin \( \beta 4 \), it became evident that classes of mutants exist. For this reason we isolated from the six villin headpiece libraries enough mutants to get a member of each class allowing rapid screening of the villin headpiece-F-actin interaction.

**An ELISA-based Method for Measuring Protein-Ligand Interactions**—The method is schematically depicted in Fig. 1.
The values are the averages of two independent measurements (i.e. independent phage preparations and independent ELISAs); and for each mutant were always measured in the same ELISA. Variation was maximally 5% for the lower absorbance values (this results in the 0.03 interval in Fig. 3) but was usually around 3% (the same applies to Figs. 4 and 5). ND, present in the library but not displayed; –, not recovered from the library.

|        | Leu<sup>17</sup> | Lys<sup>18</sup> | Lys<sup>19</sup> | Thr<sup>20</sup> | Gln<sup>21</sup> | Thr<sup>22</sup> |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Ala    | 49.2            | 80.8            | 42.4            | 30.1            | 30.9            | 30.9            |
| Val    | 23.1            | 90.4            | 33.8            | 70.3            | 74.6            | 39.8            |
| Leu    | 32.6            | 98.2            | 54.2            | 88.1            | 68.4            | 34.5            |
| Ile    | 42.2            | 102.1           | 33.6            | 58.9            | 31.5            | 39.4            |
| Tyr    | 86.5            | 53.2            | 44.7            | –               | –               | 41.9            |
| Phe    | 46.3            | 209.8           | 69.1            | 55.4            | 69.3            | 50.1            |
| Trp    | 42.6            | 201.0           | 43.5            | 105.9           | 95.5            | 18.6            |
| Lys    | 89.7            | 32.6            | 32.6            | 51.6            | 45.3            | 34.7            |
| Arg    | ND              | 33.5            | 38.0            | 157.2           | 47.7            | 29.8            |
| His    | 48.5            | 97.0            | –               | 79.9            | 30.0            | 49.9            |
| Glu    | 35.1            | 213.9           | 70.9            | 80.5            | 32.6            | –               |
| Asp    | ND              | 190.4           | 48.1            | –               | 70.1            | 23.8            |
| Gln    | 59.7            | 82.6            | 47.1            | 31.9            | 29.9            | 25.4            |
| Asn    | 161.5           | 75.0            | 34.6            | 41.7            | –               | –               |
| Cys    | 40.6            | –               | –               | 72.8            | 41.2            | 41.3            |
| Met    | ND              | 200.0           | 50.7            | –               | –               | 41.6            |
| Ser    | 43.8            | 179.1           | 36.3            | 51.3            | 34.1            | 32.7            |
| Thr    | 57.9            | 99.2            | 34.8            | 32.6            | 38.4            | 32.6            |
| Gly    | 34.0            | 77.2            | 25.1            | 84.9            | 29.3            | 25.4            |
| Pro    | 55.5            | 80.0            | 23.0            | 55.7            | 22.1            | 24.9            |

Phage Display of Conserved Actin-binding Motifs

**Table II**

### A<sub>4</sub>/A values for the different thymosin β4 mutants

The Relative Affinities of the Different Thymosin β4 Variants Are Indicative of the Mutational Tolerance—We made recombinant phages of the 103 mutants of thymosin β4, isolated from the six different libraries. All of the variants could be displayed on the phage surface, except for the Met, Arg, and Asp mutants.

Basicallly, we insert the gene of interest (or a limited library of mutants) in the gIIIp gene coding for the M13 minor coat protein pIII. We opted for the phagemid pCANTAB5E, which has been engineered for monovalent display of heterologous proteins (27), to avoid complications arising from avidity effects during measurements of relative affinity. In addition, this vector carries the coding information for the E-tag sequence, the epitope for the anti-E-tag antibody, between the gene of interest and the gIIIp gene. The presence of a M13 origin of replication in pCANTAB5E permits the packaging of the phagemid DNA when bacteria are infected with a helper phage (e.g. M13KO7) during the so-called rescue. Because we purify by cloning, we avoid laborious purification steps (note that this also avoids possible background absorbance values in the ELISA by religated phages containing no insert but expressing the E-tag). Because the system is statistically monovalent (maximally one of 2,000 recombinant phages will be divalent) (24), the resulting recombinant phages only carry one copy of the heterologous protein and can be used in an ELISA for presentation of this protein to its coated partner. In this ELISA, the measured absorbance (A<sub>4</sub>) is then proportional to the amount of protein complex formed. On the other hand, using the same phage preparations, the total amount of presented protein (A<sub>0</sub>) can be measured because only the recombinant phages carry the E-tag sequence. As derived under “Experimental Procedures” (see also Fig. 1), the formula predicts that there is a linear correlation between the equilibrium dissociation constant of the interaction and the ratio (A<sub>0</sub>/A<sub>4</sub>) of the number of recombinant phages over the amount of complex formed, provided that the amount of coated target protein is in large excess over the amount of presented protein. As an alternative for A<sub>0</sub>, one can determine the phage titer (T<sub>4</sub>) (24), assessing the sum of the recombinant and nonrecombinant phages. It is, however, important to note that only 1% of the total phage population carries a recombinant protein (31).

In a previous paper (24) we determined the T<sub>4</sub>A<sub>4</sub> values of 18 mutants of Lys<sup>19</sup> in the actin binding motif of thymosin β4. Here, we measured the A<sub>4</sub> and A<sub>0</sub> values of the same mutants in an ELISA using biotinylated actin and coated anti-E-tag antibody, respectively. We plotted T<sub>4</sub>/A<sub>4</sub> (versus A<sub>0</sub>/A<sub>4</sub> values (this study) and observed a correlation of 0.86, indicating that both measurements yield essentially the same result independent of the manner in which the amount of recombinant protein is determined (data not shown). This is with the exception of those mutants where codon usage in E. coli influences the amount of expressed fusion protein. Indeed, for thymosin β4 mutants in which Lys<sup>19</sup> had been mutated to Arg, the T<sub>4</sub>/A<sub>4</sub> values were higher for mutants with a less frequently used codon, whereas their A<sub>0</sub>/A<sub>4</sub> values were all very similar (Table I). Similarly, A<sub>0</sub>/A<sub>4</sub> values of K18Q mutants, where glutamine is encoded by TAG in the supE strain TG1 (this stop codon is only partially suppressed resulting in lower expression levels) or by the normal CAG codon, are comparable (83.7 and 82.6, respectively). Consequently, the method using titer over absorbance (T<sub>4</sub>/A<sub>4</sub>) can only be employed if one takes into account codon usage in E. coli. Thus, the purely ELISA-based method (measuring A<sub>0</sub> and A<sub>4</sub>) is superior and exclusively used in the analysis presented below.

**Correlation between the Kd and the A<sub>4</sub>/A<sub>0</sub> Values of the Different Thymosin β4 and Villin Headpiece Variants, Proof of Principle**—We predict a correlation between the Kd and the A<sub>4</sub>/A<sub>0</sub> ratio (see “Experimental Procedures”). To validate this, we determined the A<sub>4</sub>/A<sub>0</sub> values of six phage displayed thymosin β4 variants (WT, K18A, K18R, K18Y, K18E, and K19E) for which the Kd values of chemically synthesized counterparts were measured by a sequestration assay (15, 24). We also measured the Kd values of bacterially produced and purified villin headpiece wild type and mutants (K70R, K71D, K72S, and K73F) by a co-sedimentation assay and Scatchard analysis (data not shown). In both cases we plotted the Kd values versus the corresponding A<sub>4</sub>/A<sub>0</sub> values of the same phage presented mutants. There is a linear correlation of 0.99 for the thymosin β4-G-actin interaction and of 0.98 for the villin headpiece-F-actin interaction (Fig. 2). These values suggest that despite the bias favoring dissociation inherent to washing steps in the ELISA, apparent affinities can be measured, illustrating the robustness of the method.
We prepared phages displaying the villin headpiece mutant Mutilants Is Different for Interaction with F- and G-actin. Our assay. Two residues of the motif, Lys^{18} and Thr^{22}, appear to be essential because no variant has a significantly higher affinity than wild type (K18R, T20A, and T20Q have wild-type-like affinity). At the other four positions of the motif, we do observe one or more mutants that have a higher affinity than wild type (see "Discussion").

If we take into account the number of mutants displaying reduced affinity and the extent of decreased binding, simple visual inspection of the graphs in Fig. 3 allows deriving the mutational tolerance at each position in the thymosin β4 hexapeptide motif. Lys^{18} appears to be most important for the interaction with G-actin, followed by Thr^{22}, Leu^{17}, Gln^{21}, Lys^{19}, and Thr^{22}. We note that this observed tolerance agrees well with the conservation of hexapeptide motifs in various β-thymosins (16, 32, 33), i.e., more mutations are allowed in the C-terminal half. In addition, most of the naturally occurring alterations result in a good binding thymosin β4 mutant in our assay.

The Profile of Relative Affinities of the Different Villin Headpiece Mutants Is Different for Interaction with F- and G-actin—We prepared phages displaying the villin headpiece mutants and measured their relative affinities for F-actin using wild type villin headpiece as reference (Table III and Fig. 4). Because we only analyzed two-thirds of all possible mutants, we interpret our results cautiously. The first three residues of the motif appear more important because most of the analyzed mutants display significantly reduced binding, whereas at positions 72, 73, and 74 one or more mutants have a significantly higher affinity than wild type.

Although the villin headpiece is an F-actin-binding protein, we assayed the same recombinant phages for their relative affinity for G-actin in view of the observed competitive actin binding with thymosin β4 (15). For none of the recombinant phages could we observe binding when we used the same ELISA setup as for thymosin β4, i.e., with G-actin biotinylated on Cys^{774}. Possibly, the villin headpiece-binding site on actin was occluded by the neutravidin moiety because an ELISA with G-actin biotinylated on Gln^{41}, which is located at the opposite end of the actin monomer, yielded absorbance values (Table IV and Fig. 5). In general, fewer mutants bind to G-actin, and many of the ones that do bind have strongly reduced affinities for monomeric actin consistent with the preference of this module for F-actin. This is especially relevant for mutations in the first three positions of the motif. However, similar to some of the thymosin β4 mutants, these results show that very weak interactions can be measured. Intriguingly, stronger G-actin-binding variants are mainly found at position 72, whereas this is at position 73 for F-actin (compare Figs. 4 and 5).

**DISCUSSION**

An Alternative Use for Phage Display—Phage display is a widely used technique for the selection of antibodies against antigens (34) or for searching protein variants with a higher affinity than wild type (13, 35, 36). We here employ it differently, i.e., not for the purpose of selecting stronger binding mutants but rather for systematically investigating the interaction of two proteins. In combination with PCR mutagenesis and ELISA, it yields a reliable and easy way to determine relative affinities for mutants, even for those cases where partners interact weakly (we still obtained a positive signal for a low affinity interaction with a K_{d} equal to ~0.2 mM). In solid phase assays, such as the ELISA used here, there may be bias toward slowly dissociating mutants. Indeed, within the incubation time, equilibrium between the K_{d} and k_{off} values is reached, but during subsequent washing steps the K_{d} plays a major role in determining the amount of complex remaining on the plates. Nevertheless, the observed ratios of A_{0}/A (representing the relative affinities in our solid phase assay) correlate very well with the corresponding K_{d} values for the six chemically synthesized thymosin β4 variants and for the five recombinant villin headpiece variants tested (Fig. 2).

The relative affinities can be determined by two related methods. Either one calculates the ratio phage titer (T) over the absorbance (A) (24), or one determines the ratio A_{0} over A (this study). The second approach is faster, and the correlation between the K_{d} and the measured values no longer contains a proportionality factor that is dependent on a variety of instru-
mentation and environmental parameters. The most important improvement, however, is that the results become independent of the fraction of phages presenting the partner protein. An ELISA-based method for protein-ligand interaction was previously developed for the analysis of the interaction of zinc fingers with DNA (37). Similar to our strategy it relies on presenting protein in concentrations much lower than those of the target, and $K_d$ values can be determined by coating several concentrations of target ligand (preferably in 100-fold excess of the $K_d$ value). For the low affinity interactions studied here (1/$H_{9262}$ to 200/$H_{9262}$), this would have required milligram amounts of actin, which in practice is difficult to achieve (in our assays we coat nanogram amounts). Because, in our method, we measure the amount of displayed partner protein, we avoid this problem.

Several other tools and methods have been developed to study protein-protein interactions, such as multi-use peptide libraries (8) and spot synthesis (9, 10), alanine (11, 12) or cysteine (38) scanning, and two-hybrid analysis (39, 40). Our technique has important advantages over these methods. Multi-use peptide libraries and spot methods may yield very similar, but less quantitative, scan information and are limited by the length of peptides that can be chemically synthesized (generally 15–20 amino acids). There may also be size limitations for phage display, especially when using the pVIII coat protein (41); however, presenting small proteins or single domains is usually no problem unless they precipitate as inclusion bodies or are not compatible for translocation through the membrane into the periplasm. In this respect, we recovered three thymosin beta4 mutants, of which the fusion protein is not presented.

Alanine scanning is a powerful tool to study protein-protein interactions (12), but one may overlook residues participating in the interaction because this type of mutation may be rather neutral. In addition, it is not indicative for the tolerance of a functional residue (42). This is exemplified here in the thymosin beta4-G-actin analysis where, with the exception of K18A, the mutation to alanine has no dramatic effect on binding, even not for Thr^190, a residue important for the interaction. Also in two-hybrid systems large numbers of mutants can be analyzed simultaneously, but certainly the method described here allows easier quantification.

Another advantage is that the heterologous mutant proteins are purified by cloning and do not require extensive purification prior to testing their binding characteristics. Recovering and analyzing all possible mutants from a library remains time-consuming. However, this may not always be necessary.

### TABLE III

$A_0/A$ values for the interaction of different villin headpiece mutants with F-actin

| Leu$^{69}$ | Lys$^{70}$ | Lys$^{71}$ | Glu$^{72}$ | Lys$^{73}$ | Gly$^{74}$ |
|-----------|-----------|-----------|-----------|-----------|-----------|
| Ala       | –         | 353.1     | 166.9     | 183.9     |          |
| Val       | 258.6     | 333.8     | 363.8     | 274.0     |          |
| Leu       | 183.6     | 306.0     | 199.6     |          | 195.3     |
| Ile       | –         | 529.9     | 291.4     | 145.7     | –         |
| Tyr       | 229.6     | –         | –         | –         | 113.7     |
| Phe       | ND        | 437.1     | –         | 111.2     | 176.5     |
| Trp       | –         | –         | –         | 216.0     | 156.7     |
| Lys       | 195.3     | 183.6     | –         | –         | 183.6     |
| Arg       | 1412.3    | 187.3     | –         | –         | –         |
| His       | 248.1     | 352.3     | –         | 251.5     | 370.9     |
| Glu       | 193.3     | 855.7     | –         | 183.6     | 68.7      | 316.6     |
| Asp       | –         | –         | 734.4     | –         | –         |
| Glu       | ND        | –         | 216.0     | –         | 168.4     |
| Asn       | –         | 406.3     | 353.1     | 470.8     | –         | 131.4     |
| Cys       | –         | –         | 178.3     | 232.4     | –         | –         |
| Met       | ND        | –         | –         | –         | –         |
| Ser       | 180.0     | 224.0     | 235.4     | 229.5     | 324.7     | –         |
| Thr       | 360.0     | 238.0     | –         | –         | 403.4     | 680.0     |
| Gly       | 244.8     | 710.0     | 966.3     | 248.1     | 193.3     | 183.6     |
| Pro       | 270.0     | 199.4     | –         | 195.3     | –         | 126.6     |
because our results with thymosin β4 indicate that mutants appear in affinity classes. For the villin headpiece we tested two-thirds of all possible mutants, and these same groups can be distinguished, albeit that at each position of the profile is different. Consequently one can for each position analyze clones randomly, measure their relative affinity, and only sequence a few of each class. In this scenario, once optimized, the technique described here is relatively fast and can in principle be improved because several steps are adaptable to automation. As exemplified by the thymosin β4 results, our technique allows probing positional tolerance. As such, it can be applied as a screening tool prior to choosing positions that can be mutagenized when constructing libraries from which stronger binding variants will be selected.

**Probing Structural Information Using Libraries of Mutants**—One of the reasons we embarked on this project was to use the binding characteristics obtained for the extensive set of mutants to distill structural information about the mutated regions. For the discussion below, we base our interpretation solely on mutants having similar or increased affinities compared with wild type, thereby assuming they are properly folded and stable.

First, comparing the profiles per position in thymosin β4 and villin headpiece allows to discriminate between residues participating in an electrostatic interaction (Tβ4, Lys18; VHP, Lys70 and Lys71) or not (Tβ4, Lys19 and Glu20; VHP, Glu72 and Lys73). This is because we observe that some charged residues cannot be changed into any other amino acid without negatively influencing the affinity (with the exception of similarly charged side chains), and the most pronounced effect is observed for variants with an opposite charge. By contrast other positions are more tolerant for charge reversals. For thymosin β4 Lys18 an electrostatic interaction is in agreement with previous studies (14, 15), and for villin headpiece Lys70 this is evident from the NMR structure because this residue is involved in making a buried salt bridge with Asp39 (22). Intriguingly, in the latter case, substitution with Arg results in increased affinity. Perhaps the mutation to Arg yields a more stable domain enabling a better contact with F-actin. The other charged residues in villin headpiece are solvent exposed, and thus Lys71 is probably involved in an ionic contact with F-actin. Surprisingly, charge reversal at position 73 leads to increased affinity. We suggest that the third lysine residue in the motif is close to the interface with actin and that substituting it with Glu creates a new (electrostatic) interaction. Given the observation that microinjected K73E mutant still binds F-actin in vivo (30), we predict that this happens with a higher affinity.

**FIG. 5. Relative affinities of the villin headpiece mutants for G-actin.** We used the formula in the legend to Fig. 3 to calculate the relative affinities from the \( A/A_0 \) values listed in Table IV and employed the same representation as for Fig. 3. WT, WT-like activity (+), mutants that are not displayed (ND), and displayed mutants with no observed binding (–) are indicated.
Second, NMR experiments showed that the motif in noncomplexed thymosin β4 is structurally poorly defined (17, 18), whereas algorithms predict a α-helical conformation. We show that at certain positions in the motif, some substitutions yield better or wild type binders. If we correlate this with the secondary structure propensities of these mutant amino acids (43), we can speculate on the local conformation of the hexapeptide motif in the actin bound configuration. Interestingly at several of the positions glycine and proline substitutions (L17G, K19G, K19P, E21G, E21P, T22G, and T22P) are tolerated or result in better binding. Because these two amino acids are usually not present in α-helices or β-sheets, this may be an indication that the hexapeptide motif adopts a loop or β-turn structure in the thymosin β4-actin complex. These results correlate well with the NMR studies of thymosin β4 mutants in solution (i.e. not bound to actin), which show that binding requires proper termination of the N-terminal α-helix (residues 5–16) before the motif (19). Along the same lines, NMR data on villin headpiece shows a helical structure for the motif. Consistent with this is our observation that at every position in the motif (with the exception of the last residue), glycine or proline substitutions result in weaker binding than wild type. Thus, although the motifs in both proteins are rather similar in sequence, their secondary structures when bound to actin appear to be completely different, and this technique is capable of probing this. We also observed differences in binding to G- and F-actin for the villin headpiece mutants. This is most evident at the last four positions of the mutated region. Better G-actin-binding mutants at positions 71, 72, and 74 have wild type affinities for F-actin, and vice versa, stronger F-actin binders that are mainly found at position 73 behave similarly to wild type for G-actin binding. Possibly, these mutants probe conformational differences between G-actin and F-actin protomers.

Thus, using this technique one can obtain useful hints on structural information such as the type of interaction that occurs at the interface or the secondary structure required for recognition. Both are based on the tolerance of amino acids at particular positions and/or on those mutants displaying higher affinity.

**Thymosin β4 and Villin Headpiece Interact Differently with Actin**—We also show that thymosin β4 and the villin headpiece interact differently with G-actin. This is based on our observation that thymosin β4 does interact with G-actin linked via biotinylated Cys374 on neutravidin, whereas the villin headpiece shows a helical structure for the motif. Consistent with this is our observation that at every position in the motif (with the exception of the last residue), glycine or proline substitutions result in weaker binding than wild type. Thus, although the motifs in both proteins are rather similar in sequence, their secondary structures when bound to actin appear to be completely different, and this technique is capable of probing this. We also observed differences in binding to G- and F-actin for the villin headpiece mutants. This is most evident at the last four positions of the mutated region. Better G-actin-binding mutants at positions 71, 72, and 74 have wild type affinities for F-actin, and vice versa, stronger F-actin binders that are mainly found at position 73 behave similarly to wild type for G-actin binding. Possibly, these mutants probe conformational differences between G-actin and F-actin protomers.

Thus, using this technique one can obtain useful hints on structural information such as the type of interaction that occurs at the interface or the secondary structure required for recognition. Both are based on the tolerance of amino acids at particular positions and/or on those mutants displaying higher affinity.

**References**

1. Von Mering, C., Krause, R., Snel, B., Cornell, M., Oliver, S. G., Fields, S., and Bork, P. (2002) Nature 417, 399–403
2. Lo Conte, L., Chothia, C., and Janin J. (1999) J. Mol. Biol. 283, 2177–2198
3. Zunz, E. R. (2002) Biochemistry 41, 1–7
4. Vandekerckhove, J., Kaiser, D. A., and Pollard, T. D. (1989) J. Cell Biol. 109, 619–626
5. Säfström, D., Sonnich, T. R., and Elzinga, M. (1997) Biochemistry 36, 5806–5816
6. Bennett, K. L., Kussmann, M., Bjerke, P., Godzwon, M., Mikkelsen, M., Sörensen, P., and Roepstorff, P. (2000) Protein Sci. 9, 1503–1518
7. Kranz, S. W., Sonnich, T. R., Englander, D. J., and Myine, L. (1996) Curr. Opin. Struct. Biol. 6, 618–623
8. Melsen, R. H., Puijk, W. C., and Slootstra, J. W. (2000) J. Mol. Recognit. 13, 352–359
9. Frank, R. (1992) Tetrahedron 48, 9217–9223
10. Reineke, U., Vollkommer-Ergert, R., and Schneider-Mergener, J. (2001) Curr. Opin. Biotechnol. 12, 59–64
11. Wells, J. A. (1991) Methods Enzymol. 202, 390–411
12. Delano, W. L. (2002) Curr. Opin. Struct. Biol. 12, 14–20
13. Ehrlich, G. K., Berthold, W., and Bailon, P. (2000) Methods Mol. Biol. 147, 195–208
14. van Compere, K., Goethals, M., Huet, C., Louvard, D., and Vandekerckhove, J. (1992) EMBO J. 11, 4739–4746
15. Van Troys, M., Dewitte, D., Goethals, M., Carlier, M. F., Vandekerckhove, J., and Ampe, C. (1996) EMBO J. 15, 201–212
16. Van Troys, M., Vandekerckhove, J., and Ampe, C. (1999) Biochim. Biophys. Acta 1447, 323–348
17. Cmich, M., Schilcher, M., Röring, S., Voelter, W., and Holak, T. A. (1993) Eur. J. Biochem. 218, 335–344
18. Zalborsky, J., Ochskinat, H., Hannapel, K., Kalsbacher, H., Voelter, W., and Holak, T. A. (1990) Biochemistry 29, 7814–7821
19. Simeonel, C., Van Troys, M., Vandekerckhove, J., Ampe, C., and Delepiere, M. (2000) Eur. J. Biochem. 267, 3530–3538
20. Vardi, D., Chabat, A. H., Frank, B. S., Louna, E. J., Noelz, A. A., Oh, S. W., Schlescher, M., and McKittrick, C. J. (2002) Cell Motil. Cytoskeleton 53, 9–21
21. McKittrick, C. J., Matsudaira, P. T., and Kim, S. S. (1997) Nat. Struct. Biol. 4, 180–184
22. Vardi, D., Buckley, D. A., Frank, B. S., and McKittrick, C. J. (1999) J. Mol. Biol. 284, 1299–1310
23. Ho, S. S., Hunt, H. D., Horton, R. M., Pullen, J. K., and Pease, L. R. (1989) Gene (Ann.) 77, 51–59
24. Rose, S., Dewitte, D., Vandekerckhove, J., and Ampe, C. (1997) J. Prot. Chem. 16, 499–503
25. Spudich, J. A., and Watts, S. (1971) J. Biol. Chem. 246, 4866–4871
26. Takashi, R. (1988) Biochimica et Biophysica Acta 939, 943–946
27. Bass, S., Greene, R., and Wells, J. A. (1990) Proteins Struct. Funct. Genet. 8, 309–314
28. Bourdet-Sicard, R., Riicker, M., Jockusch, B. M., Guenon, P., Sansonnetti, P. J., and Tran Van Nieuw, G. (1999) EMBO J. 18, 5853–5862
29. Mertens, N., Remaut, E., and Fiers, W. (1995) EMBO J. 14, 9232–9238
30. Friederich, E., Vancompernole, K., Huet, C., Goethals, M., Finidori, J., and Vandekerckhove, J. (1998) EMBO J. 17, 16649–16658
31. Vancompernole, K., Goethals, M., Huet, C., Louvard, D., and Vandekerckhove, J. (1992) EMBO J. 11, 4739–4746
32. Van Troys, M., Dewitte, D., Goethals, M., Carlier, M. F., Vandekerckhove, J., and Ampe, C. (1996) EMBO J. 15, 293–312
33. Huff, T., Muller, C. S., Otto, A. M., Netzker, R., and Hannappel, E. (2001) Int. J. Biochem. Cell Biol. 33, 250–252
34. Hoenenboom, H. R., de Bruin, A. P., Huffen, S. E., Hoet, R. M., Arends, J. W., and Roovers, R. C. (1998) Immunotechnology 4, 1–20
35. Lowman, H. B., Bass, S. H., Simpson, N., and Wells, J. A. (1991) *Biochemistry* **30**, 10832–10838
36. Rhyner, C., Kodzias, R., and Crameri, R. (2002) *Curr. Pharm. Biotechnol.* **3**, 13–21
37. Choo, Y., and Klug, A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11168–11172
38. Doering, D. S., and Matsudaira, P. (1996) *Biochemistry* **35**, 12677–12685
39. Fields, S., and Song, O. (1989) *Nature* **340**, 245–246
40. Serebriiskii, I. G., Rhatak, V., and Golemus, G. A. (2001) *BioTechniques* **30**, 634–636
41. Kishchenko, G., Batlawa, H., and Makowski, L. (1994) *J. Mol. Biol.* **241**, 208–213
42. Jin, L., and Wells, J. A. (1994) *J. Protein Sci.* **12**, 2351–2357
43. Swindells, M. B., MacArthur, M. W., and Thornton, J. M. (1995) *Nat. Struct. Biol.* **2**, 596–603
44. Ballweber, E., Hannapel, E., Huff, T., Stephan, H., Haeuber, M., Taschner, N., Stoeffler, D., Aehle, U., and Mannherz, H. G. (2002) *J. Mol. Biol.* **315**, 613–625
45. Gribskov, M., Devereux, J., and Burgess, R. (1984) *Nucleic Acids Res.* **12**, 539–549
A Phage Display-based Method for Determination of Relative Affinities of Mutants: APPLICATION TO THE ACTIN-BINDING MOTIFS IN THYMOSIN β4 AND THE VILLIN HEADPIECE

Stefaan Rossenu, Shirley Leyman, Daisy Dewitte, Danny Peelaers, Veronique Jonckheere, Marleen Van Troys, Joël Vandekerckhove and Christophe Ampe

J. Biol. Chem. 2003, 278:16642-16650.
doi: 10.1074/jbc.M208311200 originally published online February 26, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M208311200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 45 references, 4 of which can be accessed free at http://www.jbc.org/content/278/19/16642.full.html#ref-list-1