Evolution of peptides that modulate the spectral qualities of bound, small-molecule fluorophores

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Background: Fluorophore dyes are used extensively in biomedical research to sensitively assay cellular constituents and physiology. We have created, as proof of principle, fluorophore dye binding peptides that could have applications in fluorescent dye-based approaches *in vitro* and *in vivo*.

Results: A panel of Texas red, Rhodamine red, Oregon green 514 and fluorescein binding peptides, termed here 'fluorettes', was selected via biopanning of a combinatorial library of 12-mer peptides fused to a minor coat pIII protein of the filamentous bacteriophage M13. The 'best' fluorette sequences from each of the groups were subjected to further mutagenesis, followed by a second biopanning to select a new generation of improved fluorettes. Phage were selected that had higher avidity for each fluorophore except Rhodamine red. Of these, peptides were characterized that could specifically and with high affinity bind at least one dye, Texas red, in solution. In addition, the binding of certain peptides to Texas red shifted the peak excitation and/or the emission spectra of the bound dye.

Conclusions: Peptides in the context of phage display could readily be selected that could bind to small-molecule fluorophores. The affinities of selected mutant fluorettes could be increased by mutation and further selection. Only a subset of the free peptides could bind free dyes in solution, suggesting that phage context contributed to the selection and ability of certain peptidic regions to independently bind the dyes. Future screens might lead to the creation of other dye-binding peptides with novel characteristics or Texas red derivatives with cross-linking substituents might be designed to increase the utility of the system.

Introduction

Fluorophore dyes are widely used in numerous approaches in fluorescent microscopy, flow cytometry and other detection systems because of their exquisite sensitivity and ease of use (for review, see [1] and references therein). In this study we focused on the development of fluorophore dye binding peptides, termed here 'fluorettes'. Peptides specific for different fluorophore dyes, if having sufficient affinity and specificity, could have important applications in biomaterial detection in living cells, in fluorescence resonance energy transfer (FRET) analysis (for review see [2]), and in other probes of living cell function.

Detection of proteins in living cells using fluorescence approaches has been accomplished in a variety of settings. For instance, it is possible to use ligands (or naturally derived antibodies) conjugated directly or indirectly to fluorophores as probes of the expression levels of nearly any given surface-expressed protein on living cells. In some particular cases for proteins within the cell, it is possible to use permeable ligands for individual target proteins. In such cases, the ligand is self-fluorescent, becomes fluorescent upon binding or is conjugated to fluorescent adducts. In other cases, it has been possible for many years to genetically fuse reporter enzymes such as β-galactosidase, β-glucuronidase or β-glucosidase to proteins and use a fluorogenic dye, acted upon by the reporter enzyme(s), to assay enzymatic activity on a cell by cell basis [3–5]. Other systems, including those involving β-lactamase [6], build upon those findings by applying dyes that have increased cell permeability or ratiometric fluorescent qualities, which might therefore have advantages in some applications. In recent years, proteins with inherent fluorescence, such as green fluorescent protein (GFP) [7,8], have become widespread in their application because of their ease of use, the availability of mutant proteins with differing spectral qualities in either excitation or emission, and the relative nontoxicity of the approach.

In the aforementioned cases involving fusion proteins, however, the approaches can be limited by a need to genetically fuse a relatively bulky reporter protein to the molecule under study. This can have detrimental consequences on the functionality of the protein in question or can interfere mechanistically with cellular constituents with which the protein interacts. Although it would be best to directly

measure a given target protein using a specific fluorescent dye that recognized any given target moiety, no technology exists as yet to create such reagents.

There is a need, therefore, to develop approaches that provide the building blocks for specific biomaterial detection. Combinatorial chemistry using novel fluorescent dyes might eventually provide some benefit, but research in this direction is likely to require significant resources before becoming successful. We began, therefore, to study the creation of specific dye-binding peptides that might allow us to use rational early design of potential dye-interacting scaffolds coupled to combinatorial generation of aptamers that might bind to specific dye targets and/or target proteins. The expectation is that peptide leads would be generated that would facilitate the development of more specific peptide–dye interaction systems, which could be used within living cells. The long-term goal would be to make such systems highly responsive to environmental cues for their fluorescence. For the selection of specific ligand-binding peptides, aptamer library phage display approaches are a method of choice. Such libraries are based on M13 filamentous bacteriophage [9–12], plasmid [13,14] or polysomes [15–17]. Biopanning with displayed peptide libraries revealed peptides that specifically bind to a variety of targets including enzymes [18–21], antibodies [10,13,22], lectins [23,24], nucleic acids [25,26], and small molecules such as biotin [27] and streptavidin [11,28,29]. Sufficient evidence therefore exists to indicate that peptides with affinity for proteins or small molecules could be generated, and the expectation would be that such peptides might eventually be applied to biomaterial detection.

Working with relatively small fluorophore dye molecules as a target, we applied phage display and affinity maturation [21] systems to selecting peptide domains that, in the context of phage, could bind to one or more of four different fluorophore dyes: Texas red, Rhodamine red, Oregon green 514 and fluorescein. Affinity maturation of selected phage gave rise to mutants with improved affinities over their corresponding parental peptides. A subset of the peptides was capable of binding dye in the absence of the phage scaffold. Interestingly, some of the evolved peptides, when bound to fluorophore, induced shifts in their peak excitation or emission spectra. We have termed peptides capable of binding small fluorophore dye as 'fluorettes' to denote their small nature and potential utility in fluorescence applications. Systematic application of fluorescent dye-binding peptides could prove useful in measurements of intracellular events and studies of other biomolecular interactions.

Results

Rationale behind choices of fluorophore dyes used in this study

Many fluorophore dyes are commercially available. For this study we selected four dyes by specific criteria. The first three dyes, Texas red, fluorescein and Rhodamine red, fall into one group: they have a relatively high molar extinction coefficient for absorption and excellent quantum fluorescence yields [1], appropriately overlapping spectral characteristics, and the potential for cross-quenching and/or FRET analysis. The fourth dye, Oregon green 514, related structurally to fluorescein, was chosen to determine if structure–function relationships could be discerned between fluorette peptides that bound related dyes.

The extinction coefficient and quantum yield of the fluorophores are important for sensitivity of detection and were major factors in determining choice of fluorophores in this study. Fluorescence detection by the outlined approach, at its simplest conception, is stochiometric in nature. In addition, peptide binding of the target dye might lead to some fluorescence quenching. Finally, if fluorettes can be created that can each specifically bind different dyes then it is possible to measure proximal interactions between molecules, using FRET, relative to the binding of specific dyes that have accommodating overlapping fluorescence spectra.

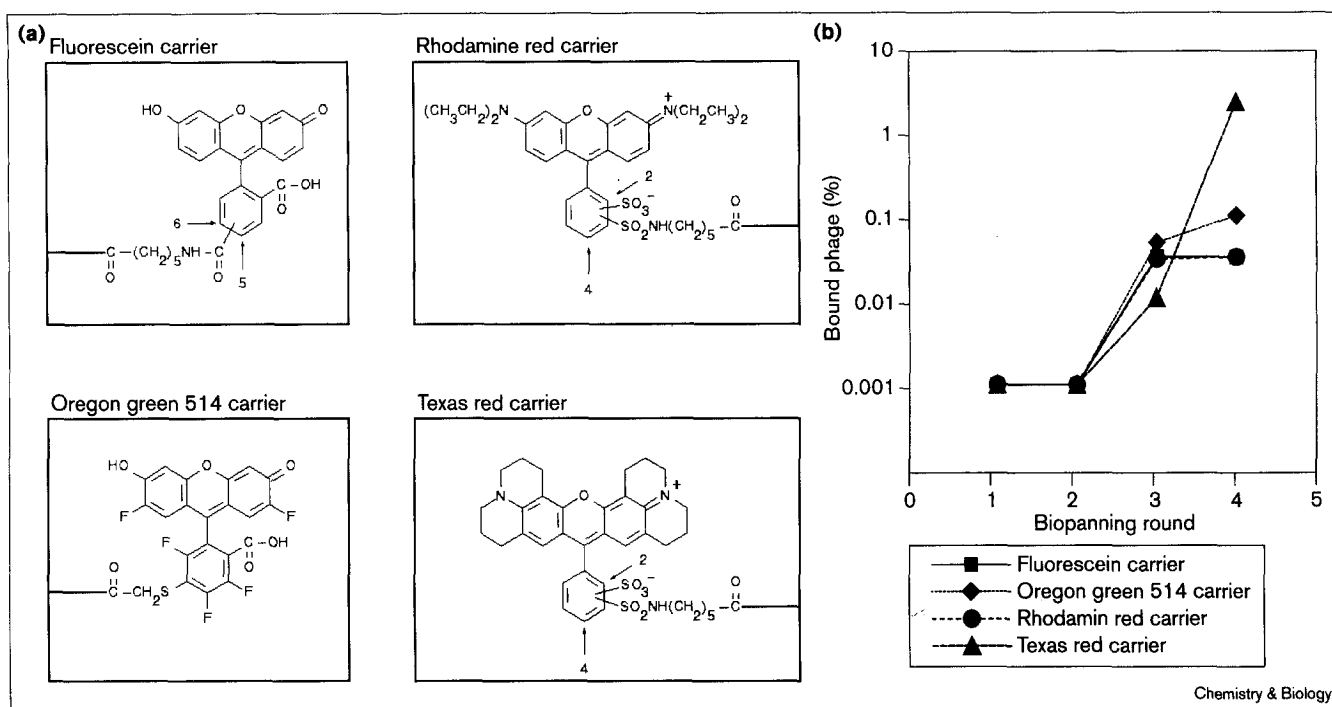### Synthesis of fluorophore-dye carriers

Succinimidyl esters of the four chosen fluorophore dyes (with a 3- or 7-atom spacer between the dye molecule and the reactive succinimidyl group) were separately and covalently linked to target beads. The chemical structures of the dye conjugates are shown in Figure 1a. Linkages were established through the formation of a stable peptide bond to Ultralink immobilized DADPA carrier beads containing a 12-atom diaminodipropylamine spacer and a terminal amino group. The resulting total spacer between the dye molecule and polymer carrier was 19 atoms long for Texas red, fluorescein and Rhodamine red conjugates, and 15 atoms long for the Oregon green 514 conjugate. This allows maximal reduction of steric hindrance for potential interactions with the large bacteriophage particles that bear the peptide libraries. After preparation, we determined that the fluorophore dye carriers contained from 0.7–1 μmoles of covalently bound dye per ml of carrier beads.

Selection for peptide aptamers that bind small-molecule fluorophores

A phage display peptide library was screened that contained a combinatorial library of 12-mer peptides fused via a short glycine linker spacer (GGG) to the amino terminus of a minor coat pIII protein (five copies per particle [30]) of the filamentous bacteriophage M13 mp19. During phage maturation, the leader secretory sequence is removed, resulting in the 12-mer peptide positioned immediately at the amino terminus of the mature protein.

Each biopanning round consisted of four sequential steps: phage binding with a fluorophore dye carrier, washing unbound phage from the beads, nonspecific elution of

Figure 1

(a) Fluorophore dye carriers. Fluorescein, Oregon green 514, Rhodamine red and Texas red activated derivatives were covalently linked to the polymer carrier Ultralink Immobilized DADPA via a 12-atom diaminodipropylamine spacer (see the Materials and methods section for details). A spacer is shown as a horizontal thick bar. Digits with arrows in the chemical structures of all fluorophore dyes, except Oregon green 514, show that 'mixed isomers' of these fluorophore dye activated derivatives were used for a coupling. (b) Biopanning with a phage display peptide library against fluorescein, Oregon green 514, Rhodamine red and Texas red carriers. A Ph.D.-12 phage display peptide library (New England Biolabs) is a combinatorial library of 12-mer peptides fused to the amino terminus of a minor coat pIII protein of the bacteriophage M13mp19 (see text). Bound phage in four biopanning rounds were calculated as a percentage of eluted phage plaque-forming units (pfu) yield versus input phage pfu (see the Materials and methods section). All experiments were performed in duplicate. Standard deviations did not exceed 19%.

bound phage and amplification of bound phage. The amplified phage were used for the next biopanning round against the corresponding fluorophore dye carrier, if necessary, until apparent enrichment for binding was observed over background. We observed a significant increase in the amounts of bound phage after 3–4 rounds of biopanning (for all of the fluorophore dye carriers; Figure 1b). This was compared with the nonspecific phage background in the first and second biopanning rounds. Eluted phage from round 4 selected against four different fluorophore dye carriers are termed TR-4, RhR-4, OG-4 and Flu-4 (Texas red, Rhodamine red, Oregon green 514 and fluorescein carriers, respectively).

The TR-4, RhR-4, OG-4 and Flu-4 phage sets were used as a source of independent phage clones that were sequenced for further analysis. The sequences from the insert regions of the phage are grouped in Table 1. Two unique sequences were found that bound to the Texas red conjugate beads, one for Rhodamine red, seven for Oregon green 514 and six for fluorescein. Note that the Oregon green 514 set and the fluorescein set shared two sequences that were identical at

the nucleotide level as well as the amino acid sequence presented. These and other issues relevant to such observations are explored later.

Phage selected against the Texas red carrier gave rise to the sequences KHVQYWTQMFYS and DFLQWKLARQKP (using single-letter amino acid code) at a 5:1 ratio (Table 1). Biopanning with the phage display library against the Rhodamine red carrier gave rise to a single phage clone, RhR401, carrying the amino acid sequence IPHPPMYWTRVF. TR-4 and RhR-4 phage sets may therefore be considered to be nearly 'pure' phage clone populations by the fourth round of selection.

We tested the binding specificity and excluded the possibility that the TR401 and RhR401 phage were selected against the polymer linker moiety rather than a fluorophore dye moiety of the dye carriers. The TR-4 and RhR-4 phage were cross-bound to Rhodamine red and Texas red carriers, respectively. Cross-binding of each phage to the inappropriate dye carrier did not exceed the nonspecific phage background binding (data not shown). The TR401 and RhR401

Table 1

Fluorophore dye specific phage clones and peptide fluorette sequences.

Fluorophore-dye carrier	Phage clone*	Phage clone frequency	Fluorette	Fluorette consensus[†]	Fluorette net charge[‡]	Fluorette hydrophobicity[§]
Texas red	TR401#	5/6	KHVQYWTQMFYS	unique sequence	+1	5/12
	TR406	1/6	DFLQWKLARQKP	(with a single exception)	+2	5/12
Rhodamine red	RhR401#	6/6	IPHPPMYWTRVF	unique sequence	+1	5/12
Oregon green 514	OG403#	4/12	HG**WDYYWDW**TAW	**YWDW**	−2	7/12
	OG401#	2/12	ASD**YWDWEW**YYS	**W(D/E)YY**	−3	7/12
	OG402#	2/12	YPNDFEWWEYYF	**YY**	−3	7/12
	OG409	1/12	HTSHISWPPWYF		0	5/12
	OG410	1/12	LEPRWGFGWWLK		+1	6/12
	OG411	1/12	Q**YY**GW**YY**DH		−1	7/12
	OG412	1/12	YMYDEYQYWNFW		−2	7/12
Fluorescein	OG402#	7/14	YPNDFEWWE**YY**F	**YY**	−3	7/12
	OG401#	2/14	ASDYWDWEW**YY**S		−3	7/12
	Flu406#	2/14	WYDDWNDWHAWP		−3	6/12
	Flu404	1/14	WHMSPSWGWGYW		0	5/12
	Flu405	1/14	HMSWWEFYLVPP		−1	6/12
	Flu413	1/14	YWDYSWH**YY**APT		−1	7/12

*Phage clones were isolated after four biopanning rounds with random combinatorial Ph.D.-12 phage display peptide library (see text). [†]Partial consensus (shown in color) for Oregon green 514 and fluorescein specific peptide fluorettes. [‡]−1 (D, E) or +1 (K, R). [§]Number of hydrophobic amino acids (A, V, L, I, W, Y and F) per total number of peptide fluorette amino acids. #Phage clone is represented in the corresponding group at least twice. Single-letter amino acid code is used.

fluorettes are therefore specific, shown by this comparison, for their respective conjugated dyes, despite the similarity of the compound core ring structures. They also had affinity for the corresponding conjugated fluorophore dyes rather than for only a polymer linker moiety of Texas red and Rhodamine red carriers or other chemical features of the carriers themselves.
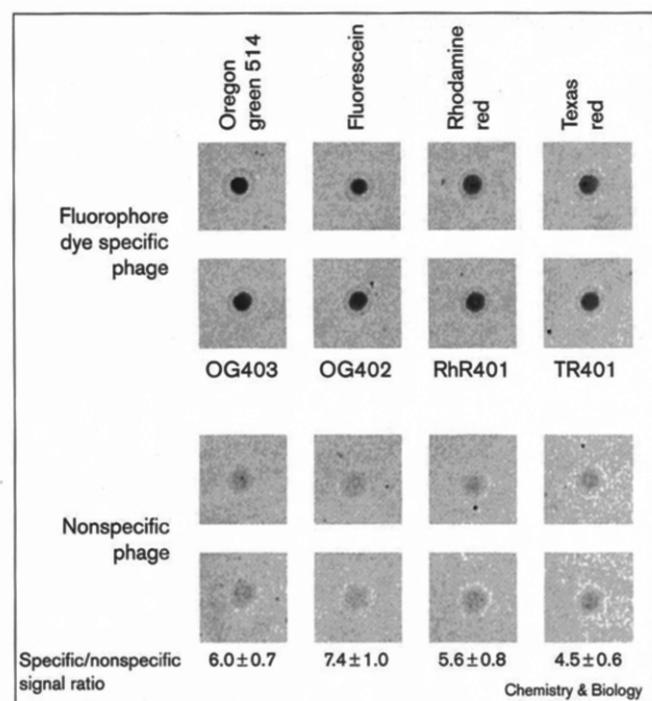
Four rounds of biopanning with the phage display peptide library against Oregon green 514 and fluorescein carriers were undertaken. Three clones were predominant in the sequenced population selected against Oregon green 514 (OG401, OG402 and OG403) and four clones were represented once (Table 1). Similarly, when selected against fluorescein, three clones predominated and others were represented only once. Notably, two of the three predominant fluorescein-specific fluorettes, OG402 fluorette (YPNDFEWWEYYF) and the OG401 fluorette (ASDY-WDWEWYYS), had the same sequence as two of the predominant, independently selected fluorettes that had been found with the Oregon green 514 carrier. As Oregon green 514 is structurally related to fluorescein and is considered a fluorescein pentafluoride (Figure 1a), independent selection of the same peptide fluorettes against Oregon green 514 and fluorescein carriers was predicted. The same fluorettes selected independently against these fluorophores possibly bind to similar domains of the Oregon green 514 and fluorescein dye molecules.

The selected phage had been selected originally as being capable of binding fluorophore dyes that had been covalently linked to a polymer carrier. The nature of carrier-cross-linked fluorophores, although useful for initial selection of phage, is inappropriate to study affinity of the phage except in relative terms. We therefore tested whether the selected phage can bind to the corresponding free fluorophore dyes in solution. Each bacteriophage particle contains five copies of pIII–fluorette fusion protein. Phage binding is therefore governed by avidity considerations. We bound free fluorophore dyes to highly concentrated phage solutions and assayed for bound fluorophore dye after precipitation of the phage. Phage–dye complexes were precipitated three times by polyethylene glycol 8000 (PEG) to remove unbound fluorophore dye, resuspended and then samples were spotted onto a single nitrocellulose filter. The filter was then scanned in a Storm 840 fluorescence detector. Nonspecific binding was determined by incubation of normalized amounts of phage particles of amplified unselected Ph.D.-12 phage display library with the corresponding fluorophore dyes under the same conditions. We found that OG403, OG402, RhR401 and TR401 phage specifically bound their respective fluorophore dyes (Figure 2). This result shows that phage selected against covalently bound fluorophore dye were still capable of interacting with their cognate free fluorophore dye in solution.
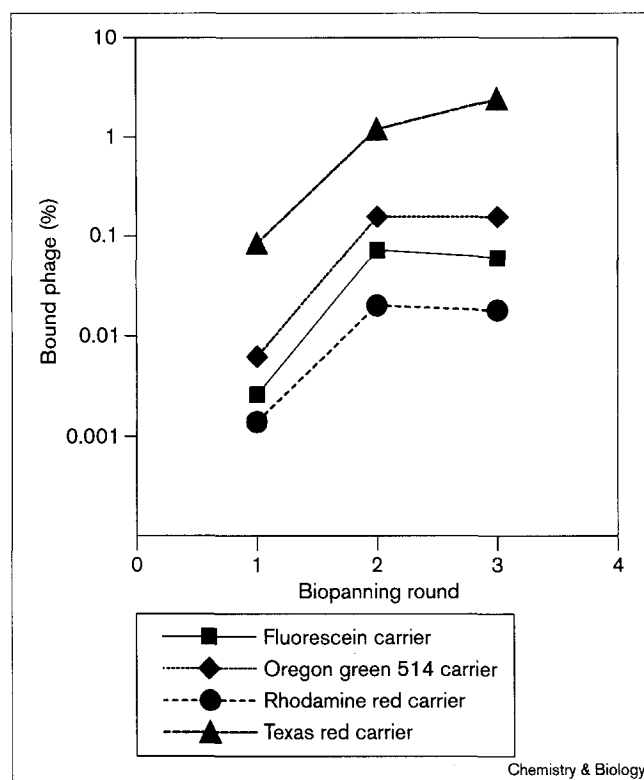
Forced evolution of higher-affinity fluorophore-binding peptides

The total number of possible combinatorially generated 12-mer peptides is equal to $20^{12} = 4.1 \times 10^{15}$. The complexity of the phage display peptide library used for biopanning against the fluorophore dye carriers was much

Figure 2

Phage–fluorophore-dye binding in solution. Fluorophore dye specific OG403, OG402, RhR401 and TR401 phage were bound to Oregon green 514, fluorescein, Rhodamine red and Texas red 'free' dyes, respectively (see text). The normalized particle amounts of amplified Ph.D.-12 phage display peptide library (nonspecific phage) were bound in solution with the same dyes as controls. Phage-fluorophore dye complexes were precipitated three times by PEG to remove unbound dye and spotted to a nitrocellulose filter. The filter was scanned on the Storm 840 scanner (Molecular Dynamics). The presented data is from a single scan and image enhancements were done simultaneously on the complete set of dye binding results using NIH Image 1.59 software. Specific/nonspecific signal ratios were quantified by densitometry of spot images using NIH Image 1.59 software (see Materials and methods for details). All binding experiments shown were completed in duplicate. Densitometry was carried out prior to contrast enhancement. Brightness and contrast values were modified to 50 and 65, respectively in Adobe Photoshop. Individual elements of the figure were arranged as a composite from the same original scan from which the binding ratios were determined.

Figure 3

Biopanning with four mutagenized phage display peptide libraries based on degenerated fluorettes against fluorescein, Oregon green 514, Rhodamine red and Texas red carriers. OG402-91CL, OG403-91CL, RhR401-91CL and TR401-91CL nonrandom combinatorial phage display peptide libraries (see text) were subjected to a biopanning against fluorescein, Oregon green 514, Rhodamine red and Texas red carriers, respectively. Bound phage in three biopanning rounds were calculated as described in a legend to Figure 1b. All experiments were performed in duplicate. Standard deviations did not exceed 21%.

smaller, containing only 1.9×10^9 clones. The library therefore represents a fraction of all possible 12-mer peptides that could have been searched for binding. We therefore sought to improve the present fluorettes. We chose to introduce mutations into the fluorette peptide sequences at the DNA level in the phage and then select for phage that displayed peptides with higher affinity (compared to the parent) for the respective fluorophore dye carrier. We chose the Texas red and Rhodamine red specific clones (TR401 and RhR401, respectively), as well as the predominant Oregon green 514 and fluorescein specific fluorette clones (OG403 and OG402, respectively, see above), for this forced evolution on the basis of initial indications of their affinities (data not shown). We set up

oligonucleotide synthesis of the corresponding fluorette-coded DNA sequences in such a manner that the mutagenesis rate was 9% for every 36 nucleotide positions of the peptide fluorette (see the Materials and methods section). Nucleotides A and C were omitted from the third position in each codon to improve the relative representation of all amino acids and limit stop codon generation. We determined the nucleotide sequences of 12 independent phage clones from each of the TR401-91CL and RhR401-91CL libraries to determine the average level of amino acid substitutions in the displayed fluorettes: 2.30 and 2.45, respectively. These experimental values correlated well to the expected theoretical values (calculated from a degeneration frequency 0.09 on the nucleotide level, see above). The nucleotide mutation rate from the other two libraries, OG403-91CL and OG402-91CL, was not determined but probably had a comparable average level of amino acid substitutions in the fluorette moiety as all four corresponding oligonucleotides were synthesized in parallel using the same batch of pre-mixed monomer nucleotides.

Table 2

Fluorophore dye specific phage clones and peptide fluorette sequences. The second generation.

Fluorophore dye carrier	Phage clone*	Phage clone frequency	Number of amino acid substitutions in the fluorette	Fluorette[†]
Texas red	TR401parent[‡]	8/16	–	KHVQYWTQMFYS
	TRS311	1/16	2	.P........T
	TRS310	1/16	2	.PA........
	TRS315	1/16	2	.N........T
	TRS313	1/16	2	......H...T
	TRS305	2/16	1	..........T
				..........T
	TRS304	1/16	2	N..H........
	TRS308	1/16	1	T..........
Rhodamine red	RhR401 parent[‡]	13/15	–	IPHPPMYWTRVF
	RhRS308	1/15	2	...R.....P..
	RhRS307	1/15	1	L...........
Oregon green 514	OG403 parent[‡]	4/16	–	HGWDYYWDWTAW
	OGS316	1/16	2	.E.E........
	OGS312	1/16	1	.E..........
	OGS303	5/16	1	..........D.
				..........D.
				..........D.
				..........D.
				..........D.
	OGS308	2/16	1	.........'.P.
				..........P.
	OGS305	1/16	1	..........T.
	OGS302	1/16	1	.....N......
	OGS301	1/16	1	Q...........
Fluorescein	OG402 parent[‡]	5/16	–	YPNDFEWWEYYF
	FluS303	1/16	4	...E.D..D..Y
	FluS302	1/16	2	........D..Y
	FluS315	1/16	3	.H..Y......Y
	FluS310	1/16	1	..........Y
	FluS307	1/16	2	.....D.....L
	FluS311	1/16	3	.TH.Y.......
	FluS312	1/16	1	....Y.......
	FluS313	1/16	1	..S.........
	FluS316	1/16	1	..S.........
	FluS304	1/16	1	.-H........
	FluS314	1/16	2	..Y........M

*Phage clones were isolated after three biopanning rounds with TR401-91CL, RhR401-91CL, OG403-91CL and OG402-91CL nonrandom combinatorial phage display peptide libraries (see text). [†]Amino acid substitutions in mutant peptide fluorette versus the corresponding parent are shown; mutant peptide fluorette sequence is shown as many times as it was found (see phage clone frequency); dots designate the same amino acid as that in the corresponding parent; dash in a second position of the FluS304 fluorette designates in-frame deletion, [‡]Original parental clones and the parental clones carrying silent mutation(s) in peptide fluorette moiety.

A key difference between the secondary biopanning and the primary biopanning with the original phage library is the strict need to maximize selection against the originating, parental fluorette. For this purpose, we significantly reduced concentrations of the fluorophore dye carrier. We also reduced the phage concentrations present during the binding steps. In addition, the binding time was reduced and the amounts of washing buffer per ml of carrier beads were increased (see the Materials and methods section). An increase in approximately 1.2–1.5 logs of magnitude in the total number of bound phage after 2–3 rounds of biopanning (compared with that of the first round) for all the fluorophore dye carriers was seen (Figure 3). Eluted

phage from round 3 were termed TRS-3, RhRS-3, OGS-3 and FluS-3. These phage represent a second generation of phage clones selected against Texas red, Rhodamine red, Oregon green 514 and fluorescein, respectively.

We isolated and sequenced a panel of independent phage clones from each of the TRS-3, RhRS-3, OGS-3 and FluS-3 phage sets to determine the sequences of the mutants (Table 2). In the Texas red selection, half of the clones sequenced encoded the parental amino acid structure, indicating successful, though partial, selection against the parental binding characteristics under these conditions. Four of the 16 TRS401 progeny clones

Table 3

Phage fluorophore dye binding affinity.

Fluorophore dye carrier	Phage clone	Fluorette*	K_d(nM)	Affinity increase (fold)[†]
Texas red	TR401 parent	KHVQYWTQMFYS	0.27	
	TRS311	.P........T	0.09	3.0
Rhodamine red	RhR401 parent	IPHPPMYWTRVF	23.0	
	RhRS308	...R.....P..	21.5	No increase
Oregon green 514	OG403 parent	HGWDYYWDWTAW	6.4	
	OGS316	.E.E........	2.4	2.7
Fluorescein	OG402 parent	YPNDFEWWEYYF	17.4	
	FluS303	...E.D..D..Y	2.7	6.5

*Amino acid substitutions in mutant peptide fluorette versus the corresponding parent are shown; dots designate the same amino acid as that in the corresponding parent. [†]Mutant peptide fluorette versus the corresponding parental peptide fluorette.

carried a conservative Ser12→Thr (S12T) substitution. Each of these substitutions occurred independently as it can be observed they are associated with additional, independent mutations, suggesting that the S12T substitution is probably an important change that enhances the affinity of the phage for the dye. Three progeny Texas red fluorettes showed the substitution His2→Pro (found twice) or Asn. It is interesting to note that clone TRS311 carried both the H2P and the S12T mutations. Fewer mutant clones (only two) were observed for Rhodamine red. The RhR401 phage clone might represent a local optimum in affinity for Rhodamine red. We found numerous in-frame deletions in the RhR401 fluorette-coded DNA sequences (about 33% of independent clones carrying large and small in-frame deletions in the fluorette moiety). This is an indicator of potential toxicity of the substituted mutant RhR401 fluorette sequences for bacteriophage growth and survival (data not shown). For Oregon green 514, multiple different peptide sequences were selected, with only two independently containing the same, nonconservative, mutation Gly2→Glu (G2E). For fluorescein, the majority of peptide fluorettes selected were also not parental

sequences. Several independent mutations were selected independently at fluorette positions 3 (Asn→Ser found twice), 5 (conservative change of Phe→Tyr found three times), 6 (conservative change Glu→Asp found twice), 9 (conservative Glu→Asp found twice), and 12 (conservative Phe→Tyr found four times). Also, certain positions appeared favored for change (2, 3 and 12) with no strong bias for the substituted residue. Evidence that underscores the importance of these changes requires the sequencing of more peptides and a fuller structure–function analysis that is beyond the scope of the current work and will be explored elsewhere.
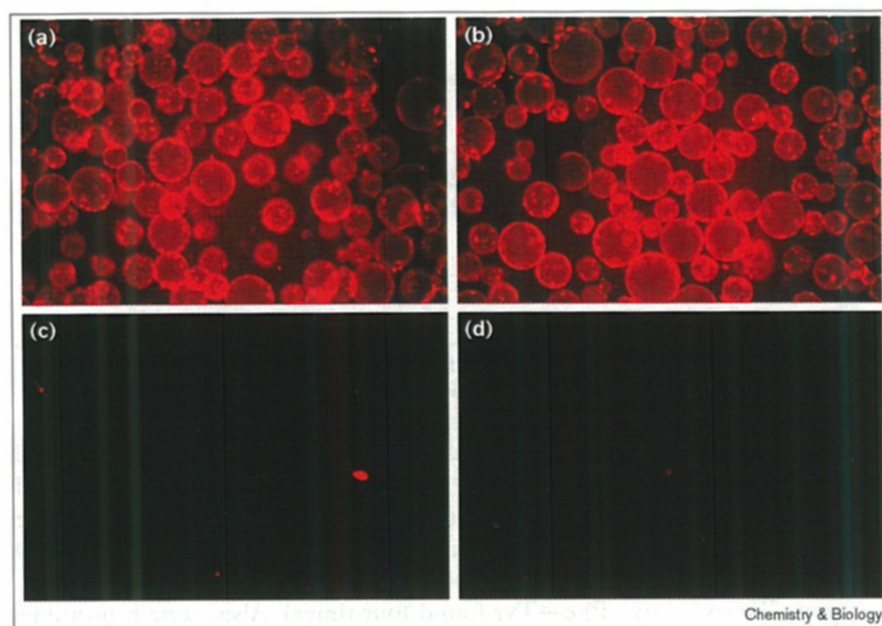
These mutations might be important for increasing the overall affinity of the peptide fluorette–fluorophore interaction. We tested one of each of the phage from the secondary screens for increases in avidity (Table 3). The Texas red progeny phage TRS311, which contained the apparently important S12T substitution, had a threefold higher avidity than the TR401 parent. The avidity of the double mutant clone RhRS308 versus the parent was not even marginally improved (Table 3). For Oregon green 514, the phage clone OGS316, which carried the G2E

Table 4

Fluorette-carrying peptides.

Fluorophore dye	Peptide*	Sequence[†]
Texas red	TR401	*KHVQYWTQMFYSGGGSAETVGGGHHHHHH*
	TRS311	*KPVQYWTQMFYTGGGSAETVGGGHHHHHH*
	TR406	*DFLQWKLARQKPGGGSAETVGGGHHHHHH*
Oregon green 514	OG403	*HGWDYYWDWTAWGGGSAETVGGGHHHHHH*
	OG401[‡]	*ASDYWDWEWYYSGGGSAETVGGGHHHHHH*
	OG402[‡]	*YPNDFEWWEYYFGGGSAETVGGGHHHHHH*
Fluorescein	FluS303	*YPNEFDWWDYYYGGGSAETVGGGHHHHHH*
–	Control[§]	*ASGSGASGSAGSGGGSAETVGGGHHHHHH*

*Peptide names reflect the names of phage clones carrying respective fluorettes except control (see Tables 1 and 2). [†]First 20 amino acids of the peptides are the same as in the pIII fusion protein of the corresponding phage clones except control (see Tables 1 and 2 and also New England Biolabs 'Ph.D.-12 Phage Display Peptide Library Kit' manual), thus, amino acids 1–12 are the fluorette portion (bold, italics) and amino acids 13–20 are the distal pIII fusion part (italics). Amino acids 21–29 contain a small GGG spacer followed by His_6 tag (shown in regular case). [‡]Specific for both Oregon green 514 and fluorescein (see Table 1). [§]Nonspecific control peptide synthesized for a specificity check.

Figure 4

Peptide–Texas-red binding. Texas red specific peptides (a) TR401, (b) TRS311, (c) TR406 and (d) nonspecific control (see peptide sequences in Table 4) were bound via His_6 tag to cobalt-ion-containing Sepharose beads. The peptide-coated beads were washed and incubated with 0.5 µM Texas red in TBS for 1 h at room temperature followed by several washings of beads in order to remove unbound dye (see the Materials and methods section for details). Fluorescent and nonfluorescent control beads were photographed on fluorescent microscope Axiophot (Zeiss) using a Rhodamine red/Texas red filter with the same time exposure for every sample. All binding experiments were accomplished in three parallel experiments and results of a single experiment are shown.

substitution, had a 2.7-fold higher avidity than its parent. The multiply substituted clone FluS303, which contained two putatively important changes, had a 6.5-fold increase in relative avidity. Peptides can therefore be matured to higher avidity, probably as a result of corresponding affinity increases for the individual peptide fluorette against its respective dye.

Binding of peptides to fluorophores in solution

Creation of peptide fluorettes that bind fluorophore dye when not in the full context of the pIII fusion bacteriophage protein is a goal of this study. We prepared synthetic peptides corresponding to the sequence of the selected fluorette region. We added to the peptide eight amino acids derived from the adjacent sequence of the pIII protein, which would accommodate any partial contributions of context from the pIII protein sequence. These were followed by a short GGG spacer and His_6 tag (Table 4). We synthesized peptides from the original library screening that had been capable, in solution, of specifically binding each of the three dyes (Texas red, Oregon green 514 and fluorescein). The Texas red peptides chosen were TR401 and the peptide from its higher avidity progeny phage TRS311; a peptide corresponding to the unrelated Texas red primary clone TR406 was also synthesized. The Oregon green 514 clones OG401 and OG403 were synthesized to test, in part, the observation that both contained a common motif, YWDW, that might represent a common binding motif for Oregon green 514. Note that the OG401 clone also bound to fluorescein in the original screening. The other clone that bound to both Orgeon green 514 and fluorescein in the primary screen,

OG402, was synthesized. The peptide derived from a secondary screen against fluorescein, FluS303, which demonstrated a 6.5-fold higher affinity, was also synthesized. No peptide for Rhodamine red was synthesized.
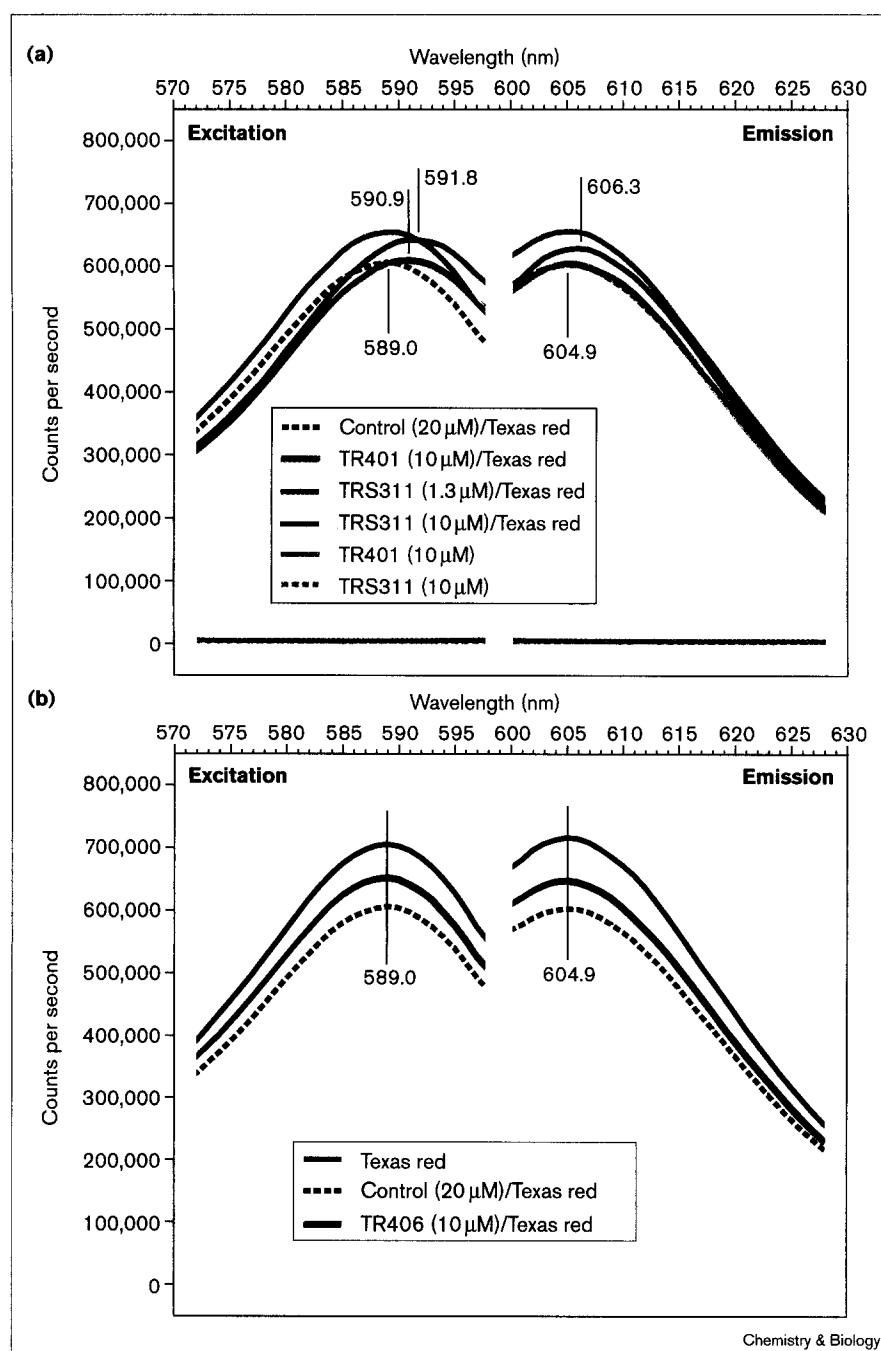
Peptides were bound to cobalt-ion-coated Sepharose beads via the His_6 tag and the resultant peptide-coated beads were incubated with corresponding fluorophore dyes, washed and visualized for dye binding. Most peptides failed to bind dyes to any detectable degree. Texas red peptides TR401 and TRS311, however, showed significant binding to Texas red (Figure 4), whereas they bound to neither Oregon green 514 nor fluorescein (data not shown). Binding of these dyes is therefore specific. TR406 did not bind to Texas red, however (Figure 4). Lack of dye binding by the other peptides to their respective dyes might be due to low peptide affinity for cognate dyes and/or because the binding requires contributions from the pIII fusion protein not present in these peptides.

The above test only checks for bound dye that remains fluorescent at the wavelengths tested. Binding of fluorophore dyes to specific peptides might result in changes in the fluorescence spectra of bound dye versus the spectra of free dye, or one might observe an increase or quenching of fluorescence. We mixed concentrated peptide with 50 nM of the corresponding dye in solution and determined the excitation and emission spectra of the resulting mixture. No detectable changes in peak excitation nor emission were observed for peptides selected against Oregon green 514 or fluorescein (data not shown), nor for TR406, nor for a control peptide (Figure 5b).

Figure 5

Peptide–Texas red binding. **(a,b)** Excitation
and emission spectra. Peptides at the noted
concentrations were incubated with Texas red
(50 nM), or without, in 0.6 ml of TBS for 1 h at
room temperature. Samples were scanned on
a spectrofluorimeter (SPEX Fluoromax (Jobin
Yvon-SPEX Instruments Co.). Peak excitation
or emission positions are shown in the figure
using vertical bars. Excitation and
excitation/emission peak shifts for TR401
(10 µM)/Texas red and TRS311
(10 µM)/Texas red complexes, respectively (a)
were reproducible in three independent
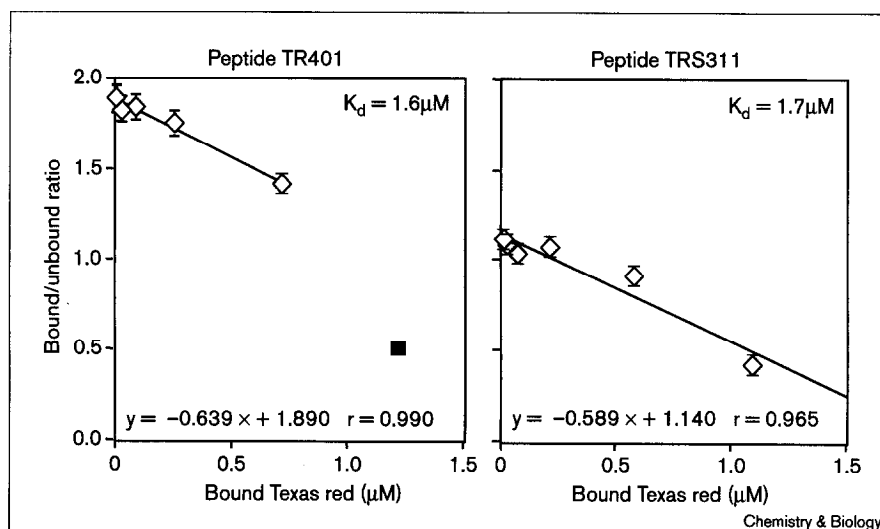experiments. The results of a single
experiment are presented.

(a) Wavelength (nm)

Counts per second

- - - - Control (20 µM)/Texas red
──── TR401 (10 µM)/Texas red
──── TRS311 (1.3 µM)/Texas red
──── TRS311 (10 µM)/Texas red
──── TR401 (10 µM)
- - - - TRS311 (10 µM)

Excitation   590.9 591.8   Emission   606.3
589.0   604.9

(b) Wavelength (nm)

Counts per second

Excitation   589.0   604.9   Emission

──── Texas red
- - - - Control (20 µM)/Texas red
──── TR406 (10 µM)/Texas red

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The peak positions of the excitation and emission spectra of soluble Texas red bound to 10 µM TR401, however, were shifted relative to Texas red alone or as compared with a mixture of Texas red and a nonspecific control peptide (Figure 5a). A peak excitation shift (+1.9 nm) with TR401 was observed. The peptide was checked against fluorescein as a nonspecific control and showed no shift in the fluorescein excitation or emission spectra. An approximate peak excitation shift of +2.8 nm was effected upon dye binding with TRS311. Interestingly, although TR401 had a shift in

its peak excitation, no emission shift change was elicited upon binding fluorophore. TRS311 did manifest a significant (+1.4 nM) peak emission shift in its spectra, however. We checked all other excitation and emission wavelengths and the changes observed correspond to a largely global shift in the spectra. No other significant changes in local optima were observed (data not shown).

Interestingly, the TR401 peptide is the progenitor of the TRS311 peptide. The two substitutions H2P and S12T

Figure 6

Peptide–Texas-red binding. Scatchard plots and dissociation constants. Texas red in different concentrations was bound to the TALON beads loaded by TR401 or TRS311. Beads were then washed and amounts of bound Texas red were determined (see the Materials and methods section for details). Standard Scatchard plots were built by a 'Linear Method' of 'Curve Fit' option (Cricket Graph 1.53 software package). Equations at the bottom of each graph show automatically calculated linear functions; r, coefficient display. Open diamonds present a linear range of Scatchard plots; the filled square in the left graph is out of a linear range and was not counted in the calculation. Dissociation constant values for TR401 and TRS311 are shown at the right top corner of each graph.

therefore result not only in an increased binding, but also in an apparent differences in the excitation spectra elicited. Moreover, although both show a shift in the excitation only TRS311 had a peak emission spectra shift. One or both of the two substitutions, H2P or S12T, is therefore critical for shifting the spectra of the emission profile.

We attempted with these findings to obtain an affinity measurement for the TRS311 peptide. Reduction of the concentration of TRS311 from 10 µM to 1.3 µM reduced slightly, but not completely, the change in peak excitation position (Figure 5a). The narrow dynamic range of the shift and the broad emission spectra gave us a limited ability to obtain an approximate measure of the affinity. Affinity measurements calculated roughly from these results place the affinity of the peptide for the fluorophore in the micromolar range. We therefore directly measured the Texas red binding affinities of TR401 and TRS311. The dissociation constants were determined to be 1.6 µM and 1.7 µM, respectively (Figure 6). In addition, we were able to effectively compete Texas red that had been pre-bound to beads by an excess of soluble peptides (data not shown). This implies that a peptide in the context of a Sepharose bead is not critical for dye binding. We can therefore conclude that we have successfully selected for peptides with sufficient affinity to bind small-molecule fluorophore dyes in solution. Also, the binding is specific and the spectral qualities of the dyes can be modulated dependent upon modifications of the bound peptide.

Discussion

The objective of this work was to devise, or select, peptides consisting of natural amino acids that were capable of binding small organic fluorophore-dye molecules. In so

doing, we expect, eventually, to create small peptide epitope motifs that can label individual molecules in living cells noncovalently with fluorescent tags. For this study, we chose four fluorophore dyes of the many available based on several criteria. First, high sensitivity of fluorophore dye detection is proportional to a dye's $\varepsilon_{max} \times QY$, where ε_{max} is the molar extinction coefficient for absorption and QY is the quantum yield for fluorescence [1]. Second, different fluorophore dye spectral characteristics were desired that might allow us to detect different dyes simultaneously, if necessary. Third, at least one pair of chemically related fluorophore dyes was needed to assay structure–function relationships for peptides that bind related compounds. The four fluorophore dyes used meet the first requirement possessing the highest ranks of $\varepsilon_{max} \times QY$ amongst available dyes [1], as well as a second requirement of appropriately overlapping absorption/emission maxima for Texas red, Rhodamine red, Oregon green 514 and fluorescein. Oregon green 514 and fluorescein meet the third requirement (Figure 1a).

Speculations on the chemical nature of peptide fluorettes and their dye-binding properties

In any set of comparisons where evolutionary considerations arise it is often useful to look for convergence of structural features related to function. Interestingly, sequences of the clones from the primary screens have several common motifs that would not be expected to arise simultaneously and are likely to be correlated to the fluorophore structures with which they interact. For example, TR401 and RhR401 phage share a common fluorette YWT motif. They may, therefore, bind a central portion of Texas red and Rhodamine red dyes that is chemically identical but likely to be structurally dissimilar (Figure 1a). The TR401 and RhR401 phage did not cross-bind to Rhodamine red carrier

or Texas red carriers, respectively. This suggests that sharing of YWT motif may be a coincidence or that the YWT motif is important but other peptide determinants can dominate the binding potential. The TR401 phage clone could bind Texas red conjugated to beads with apparent subnanomolar K_d, whereas the RhR401 clone was 100-fold less avid for Rhodamine red. Attempts to evolve the RhR401 met with no success. Perhaps, then, the YWT motif is capable of securing an interaction with Texas red and Rhodamine red, but structural constraints prevented affinity maturation against the Rhodamine red fluorophore. Although the statistics with these clones are insufficient to make a final statement it should be noted that none of the peptides in the secondary screen resulted in substitutions at the YWT motif.

Two of three major Oregon green 514 specific fluorettes were also independently selected in a screen against the fluorescein carrier (Table 1). This finding of peptides common to both Oregon green 514 and fluorescein was expected because these two fluorophore dyes are closely related (see above and Figure 1a). This also demonstrates that the selections were capable of scanning all potential phage in the population, demonstrating saturation of the screen. It will be interesting to study further how these phage-associated peptides bind these two related compounds. Interestingly, the OG401 peptide sequence shares a YWDW motif with the OG403 peptide. Although we did not undertake site-specific mutagenesis of this region, it is notable that only a single clone from the secondary Oregon green 514 screen underwent substitution in this motif (OGS302). One motif, WDYY, in OG403 was substituted in a secondary screen phage to the sequence WEYY, a conservative change. The sequence WEYY is also found in the primary screen isolate OG402 and also makes this region of the peptide more similar to a fluorescein primary isolate Flu405, which contains WEFY. Other recurring motifs were evident but their nature, beyond a high apparent hydrophobic content, remains undetermined and awaits future analysis.

The fluorophore dyes found in this study have two important chemical features: high core hydrophobicity of the organic constituents and a positive or negative electron density of dye polyaromatic systems. For binding, the amino acid composition of most peptide fluorettes is mostly compatible with these features. The TR401 and RhR401 fluorettes possessed a slightly positive net charge (+1), largely in accordance with a slightly negative electron density of Texas red and Rhodamine red polyaromatic systems. The quaternary amine of the Rhodamine red and Texas red dyes, however, is not compatible with the positive net charge of the peptides. It could be accounted for by local interactions of the peptides with negatively charged regions of the dyes while having neutral or negatively charged residues that shield the amine. The fluorette

hydrophobicity exceeded a random level of protein hydrophobicity. Interestingly, the hydrophobicity of the TR401 and RhR401 fluorettes slightly exceeded a random level of protein hydrophobicity. The probability of finding seven randomly distributed hydrophobic amino acids A, V, L, I, W, Y and F (considering the NNK nucleotide bias in the creation of the library) is 11/31 or 36%. The frequency across the three clones for the Texas red and Rhodamine red group was 42%. For the Oregon green 514 and fluorescein clones the percentage is higher (54%). Neither of these characteristics was changed in most selected TR401 progeny fluorettes, suggesting they are important (Table 2).

Finally, Oregon green 514 and fluorescein specific fluorettes (two of them are dually specific, see above and Table 1) showed both a significant net negative charge (–2 or –3) and hydrophobicity (≥ 50% of fluorette amino acid content was hydrophobic). This is in accordance with overall fluorophore dye hydrophobicity and a high positive electron density of dye polyaromatic systems, respectively. No progeny of the OG402 parent changed the parental net charge –3, whereas seven of the 12 progeny of OG403 parent acquired the same net charge compared with a net charge of –2 for the OG403 parent. The FluS303 fluorette carried four homologous substitutions and three of them were either Asp→Glu or Glu→Asp substitutions, so did not change the net fluorette charge of –3. Glu→Asp substitutions were also found in the same position amongst other fluorettes (Table 2). The fourth FluS303 fluorette homologous substitution F12Y was found as many as four times in the population. The FluS303 clone appeared to be 6.5-fold more avid than the parent OG402 (Table 3). The importance of carrying of E9D and F12Y mutations in FluS303 fluorette is strengthened by a finding of a double mutant FluS302 fluorette carrying the same pair of mutations. Another homologous substitution F5Y found three times, as well as substitution of asparagine four times in a third position (two of them were N3S; Table 2), could also be important. These results strongly suggest that a net charge of –3 might be important for functioning of the major Oregon green 514 and fluorescein specific fluorettes. Hydrophobic and negatively charged amino acids are interspersed in these peptide fluorettes rather than clustered (Table 1). Although substitutions occurred with aspartic acid and glutamic acid, there is no clear answer as to whether these negatively charged residues specifically interact with the dye compounds or they just serve to neutralize the high positive electron density of the polyaromatic systems of the bound compounds. Nonhomologous substitutions were also observed. The A11D substitution in the OGS303 fluorette was found as many as five times in the mutated population, and it also increased the fluorette net negative charge. Thus, a net negative charge of –3 might be important for Oregon green 514 and fluorescein specific fluorettes. Other mutations in the same position (A11P in OGS308 fluorette was found twice and A11T in

OGS305 fluorette) are less informative, however, but might indicate that alanine is detrimental to binding affinity. The deprotonated hydroxyl group in the fluorescein aromatic system is not compatible with a net negative charge of the peptides, however. As before, negatively charged determinants of the peptides could interact with positive density regions of the fluorophores, leaving the charged region corresponding to the hydroxyl group to interact with neutral residues or occasional positive residues found in most of the peptides. The division of labor between binding and neutralizing/solubilizing residues therefore remains to be further investigated as do the relative contributions of certain individual residues.

Evolution of modified peptides that bind small organic molecules

We were able to evolve phage-selected peptides to have higher affinity for target fluorophores and, at the same time, coincidentally, find a subset of these that could bind fluorophore dyes in solution, and, in so doing, modestly change the spectral character of bound dyes. The selection procedure for fluorophore-binding peptides can now be modified, on the basis of the following considerations, to select more directly for higher-affinity peptides that are more likely to be capable of binding independently of the selection scaffold and with additional capabilities in modulating the fluorescent character of the bound dye. In the original searches the requirement for an amplification step selected for those phage with the best replicating capacity. In this 'biathlon' the winning phage must show high scores in both binding and replication properties to survive. Certain insertions into the M13 bacteriophage pIII gene have been shown to decrease phage survival compared with more fit phage [31]. For instance, the RhR401 clone could not, apparently, be evolved in the secondary second biopanning, whereas the parental RhR401 phage had a comparable replication capacity with the amplified Ph.D.-12 phage display peptide library (data not shown). Failure of the RhR401 fluorette to evolve is therefore probably linked to a poor survival of most mutant RhR401 fluorettes. Insufficient mutagenesis can be excluded because the average amount of amino acid substitutions in the RhR401-91CL phage library was directly measured and because ~70% of recovered parental clones carried silent mutation(s) in a fluorette moiety (data not shown). Consideration of whether one has reached a local optimum is therefore a requirement to know whether or not to proceed with a screen further.

In addition, the stringency of counter selection against the parent is an important issue because an increase in affinity of new 'improved' fluorettes is largely unknown. Too mild a counter selection might not eliminate a parent at all, substantially blocking a selection of more avid fluorettes, particularly if their affinities do differ only slightly from that of a parent. Too stringent a counter selection might

well eliminate a whole population entirely, however. Our conditions for counter selection against parental phage seemed to be satisfactory judged by a yield of new Texas red, Oregon green 514 and fluorescein specific fluorettes. The parental clones partially recovered, however, accounted for 25–50% of the populations in contrast to the results observed with Rhodamine red specific fluorette.

Selection of mutant fluorettes was a key part of this study because the mutants allowed us to reveal possible important mutations resulting in fluorette improvement and compare the affinities of the mutants to those of the respective parents. For example, the second generation of Texas-red-specific fluorettes showed S12T and H2P substitutions were important, whereas new fluorescein-specific fluorettes strongly indicated the significance of F12Y and F5Y substitutions. If the fluorette is actually being improved it must show a higher affinity for a fluorophore dye than that of the parent. The affinities of chosen selected mutant Texas red, Oregon green514 and fluorescein specific clones were increased several-fold over the respective parents (Table 3). The mutant Texas red specific TRS311 clone had the highest in avidity amongst all tested clones in our study with a K_d value in 100 pM range. It went on to yield the peptides with the highest affinities (Figures 4 and 5).

Why did not more of the phage yield peptides that could bind fluorophores in a soluble context? Selection for peptide binding is done under avidity considerations in the context of phage proteins. There might some aspect of phage pIII protein that contributes in some manner to binding or aromatic ring structures. Also, peptide binding in these cases is using rotationally variable polymers that might depend upon a support interaction from nearby pIII protein. In this regard, there is the issue of the conformational flexibility of the peptides and what is likely to yield lower affinity interactions with target molecules. Provisions in future selections should therefore be made for conformationally constrained peptides with the possibility of binding pockets. We have recently succeeded in the generation of a scaffolding structure using short dimerizing motifs and a variable loops region that can be independently expressed in mammalian cells or at the cell surface (J. Caldwell, B. Peelle, M.N.R., D. Payan, D. Anderson, G.P.N., unpublished observations). Searches basing selection on such structures could yield peptidic domains that are more likely to be free of the need for support interactions from associated proteins. As we can also express such structures freely within cells (J. Caldwell, G.P.N, unpublished observations) there is no major concern about contributions from support proteins such as pIII because the selections can be done within cells directly for fluorophore-binding enhancements or fluorescence modulations. Additionally, it is possible now that a peptide has been made capable of having reasonable affinity for a target fluorophore to modify the fluorophore

with chemically reactive groups. If the reactive adducts, such as light-activatable cross-linkers, are appropriately positioned, one can readily design or select for peptide residues capable of permitting covalent cross-linking of the fluorophore to the target.

Two papers [32,33] that impact upon our work describing methods for sequence-selective peptide-dependent detection of nonfluorescent and fluorescent dyes have been published recently. Both are significantly different from our results but bear consideration. In the manuscript of Chen et al. [32] tripeptides were selected that contain non-natural amino acids (combinations of N-acetylated D- and L-amino acids) capable of binding specialized quenched fluorophore dyes. Binding of the peptide to these dyes displaced the quenching adduct, allowing the compound to be fluorescent. The binding was undertaken in organic solvent rather than in physiological solution. Binding affinity of these tripeptides for fluorophore dyes was sufficiently low (K_d values in the ten micromolar range for the most avid tripeptide) or relatively nonspecific. In our case, peptides were selected in physiological solvent against known dyes that can be made soluble in physiological conditions that are compatible with living cells. Also, as our goal remains to express these peptide sequences as 'tags' within cells, the peptides selected use natural amino acids. Finally, the fluorophore dyes we used can be loaded into eukaryotic cells where they might be used to detect the presence of peptides that have high affinity for the dyes. The second report [33] used a predesigned cysteine-rich peptide that is a target for a reactive arsenic-containing modified fluorophore. In this work, the designed peptides are exposed to the modified fluorogenic organoarsenical compound and, by virtue of a relatively rapid interaction with reactive cysteines, covalent labelling of the target cysteine-rich peptide is achieved. This could be carried out with an overexpressed protein carrying the designed cysteine-rich peptide as a fusion domain. To limit toxicity of the arsenical compound the authors [33] propose use of an antiarsenical antidote to be administered to the cells during the staining process. Low level reactivity of the arsenical with cellular constituents could lead to detrimental effects on target cells, however, and, indeed, the EDT vicinal dithiol (1,2 ethanedithiol) by itself might influence redox-dependent systems. The latter approach might therefore be limited in some circumstances.

Significance

We report here the creation of a panel of fluorophore dye binding peptides that we termed 'fluorettes'. Our finding of high avidity Texas red, Rhodamine red, Oregon green 514 and fluorescein specific peptide phage suggests they could eventually be used in approaches involving fluorophore dyes, for example, in fluorescence resonance energy transfer analysis. The fluorettes need further enhancements to become broadly useful, however. Most importantly, higher-affinity peptides must be created, as well as more efficient approaches to directly screening for peptides capable of modulating more directly the fluorescence character of bound dyes. Specifically, the Texas red fluorettes hold the most immediate promise because it is likely that we will be able to readily evolve them to create higher affinity peptides and devise simple approaches to covalently cross-link the fluorette peptide to Texas red upon binding. We expect that the creation of such unique fluorophore dye-binding peptides that have high specificity for binding to and specifically modifying the fluorescent qualities of fluorophore dyes will have far-ranging applications in biomedical analytical approaches in living single cells.

Materials and methods
Bacterial host strains and bacteriophage
Escherichia coli ER2537 strain [F' lacIq Δ(lacZ)M15 proA+B+ /fhuA2 supE thi Δ(lac-proAB) Δ(hsdSM-mcrB)5 (rk⁻ mk⁻ mcrBC⁻)] and E. coli TG-1 strain [supE thi-1 Δ(lac-proAB) Δ(mcrB-hsdSM)5 (rk⁻ mk⁻) (F' traD36 proAB lacIqZΔM15)] were purchased from New England Biolabs and Stratagene, respectively, and used as bacteriophage M13mp19 hosts. All bacteriological techniques were performed as described previously [34,35].

Phage DNA sequencing
Phage single-stranded DNAs (ssDNAs) and double-stranded DNAs (dsDNAs) were purified as described previously [34]. Fluorette-coding portions of the DNA and adjacent DNA regions were sequenced with −96 gIII sequencing primer CCCTCATAGTTAGCGTAACG (New England Biolabs) using an Applied Biosystems 391 automated DNA sequencer.

Synthesis of fluorophore dye carriers
The polymer carrier UltraLink Immobilized DADPA (capacity − 45 µmoles of free amino groups per ml of beads) was purchased from Pierce. Activated derivatives of fluorophore dyes: 6-(fluorescein-5-(and-6)-carboxamido)hexanoic acid, succinimidyl ester (5(6)-SFX), 'mixed isomers'; Oregon green 514 carboxylic acid, succinimidyl ester; Rhodamine red-X, succinimidyl ester, 'mixed isomers' and Texas red-X, succinimidyl ester, 'mixed isomers' were purchased from Molecular Probes. Covalent coupling of activated derivatives of fluorophore dyes to a polymer carrier was performed in accordance with both manufacturer's protocols, with some modifications. Briefly, 1 ml of each dye derivative (5 mg/ml in anhydrous dimetylformamide) was separately mixed with 15 ml of 50% slurry of UltraLink Immobilized DADPA in 0.2 M sodium bicarbonate buffer, pH 8.3 (the coupling yield was at least 95% and, consequently, four fluorophore dye carriers contained 0.7–1 µ Moles of covalently bound dyes per ml of beads), unreacted amino groups of the carriers were extensively acetylated by an addition of 18-fold molar excess of acetic acid N-hydroxysuccinimide ester (Sigma) followed by hydroxylamine treatment of the fluorophore dye carriers in order to destroy any unstable intermolecular intermediates of fluorophore dyes via their hydroxyl groups (for fluorescein and Oregon green 514 carriers only). Finally, fluorophore dye carriers were quenched in 0.75 M Tris-HCl, pH 8.7, loaded to the columns and extensively washed in high salt buffer, 50 mM Tris-HCl, pH 7.5 + 1 M NaCl followed by a storage buffer, 50 mM Tris-HCl, pH 7.5 + 150 mM NaCl (TBS) + 0.05% sodium azide. Fluorophore dye carriers were stored as 50% slurries at 4°C.

Biopanning with the Ph.D.-12 phage display peptide library
The Ph.D.-12 phage display peptide library (based on modified M13mp19 bacteriophage, 1.9 × 10⁹ independent transformants) as a part of the Ph.D.-12 phage display peptide library kit was purchased from New England Biolabs. All procedures were carried out at room

temperature unless noted. Binding step: 0.15 ml of centrifuged fluorophore dye carrier beads were blocked with 3 ml of TBS + 2 mg/ml BSA (blocking buffer) for 1.5 h with gentle rotating, beads were washed with 15 ml TBS + 0.1% Tween-20 + 0.5 mg/ml BSA (binding buffer), centrifuged, mixed with 1.8×10^{11} plaque-forming units (pfu) of the Ph.D.-12 phage display peptide library (~95-fold library size) in 3 ml of binding buffer and suspension was gently rotated for 4 h. Washing step: beads suspensions were centrifuged and beads were transferred to the microcolumns and slowly washed by 100-fold beads volume of TBS + 0.1% Tween-20. Elution step: the bound phage were nonspecifically eluted by 1 ml of 0.2 M glycine-HCl, pH 2.2 + 1 mg/ml BSA (elution buffer) for 10 min and the eluates were immediately adjusted to a neutral pH by 0.15 ml of 1 M Tris-HCl, pH 9.1. Bound phage yields were determined by a titration of the eluates on the ER2537 host strain. Amplification step: all bound phage (1.1 ml) were added to 27 ml of 1/100 diluted overnight ER2537 culture and amplified for 4.5 h at 37°C with vigorous shaking. The amplified phage were precipitated from cell supernatants by 1/5 V of 20% PEG + 2.5 M NaCl at 4°C, re-precipitated again and, finally, suspended in 0.5 ml TBS + 0.05% sodium azide. The resultant ~50-fold concentrated amplified phage were stored at 4°C and their titers usually were in the range $0.5–1.5 \times 10^{13}$ pfu/ml. For long-term storage amplified phage were adjusted to 50% glycerol and stored at -20°C.

The amplified phage from round 1 were further selected against the corresponding fluorophore dye carrier. The next biopanning rounds were performed in the same manner as round 1, except that: input phage in the binding reaction were increased to 4.5×10^{11} pfu, time of binding was reduced to 2 h in round 4, Tween-20 concentration in a washing buffer was increased to 0.2% in round 3 and 0.4% in round 4, eluted phage from last round 4 were not amplified, but rather served as a source of independent phage clones used for sequencing.

Construction of mutagenized combinatorial phage display peptide libraries

Four minus-strand oligonucleotides containing degenerated fluorette-coding sequences were synthesized (Protein & Nucleic Acids Facility, Stanford University Medical Center): TR401-91CL: CTCCCCTTCG-GCCGAACCTCCACC*AGAATAAAACATCTGCGTCCAATACTGCAC-ATGCTT*AGAGTGAGAATAGAAAGGTACCACTCTCCC; RhR401-91CL: CTCCCCTTCGGCCGAACCTCCACC*AAACACACACGAGTCCAATAC-ATAGGAGGATGCGGAAT*AGAGTGAGAATAGAAAGGTACCACTCTC-CC; OG402-91CL: CTCCCCTTCGGCCGAACCTCCACC*AAAATA-ATACTCCCACCACTCAAAATCATTCGGATA*AGAGTGAGAATAGAA-AGGTACCACTCTCCC; OG403-91CL: CTCCCCTTCGGCCGAAC-CTCCACC*CCAAGCAGTCCAATCCCAATAATAATCCCACCCAT*GAG-AGTGAGAATAGAAAGGTACCACTCTCCC (nucleotide in a regular case shows no degeneracy; boldfaced nucleotide designates 91% of shown nucleotide and 3 × 3% of each of the other three nucleotides; boldfaced and italicized nucleotide designates 91% of A or C and 9% of C or A, respectively). Degenerated minus-strand oligonucleotides were separately annealed to the plus-strand oligonucleotide GGGAG-AGTGGTACCTTTCTATTCTCAC, partial duplexes were filled by T4 DNA Polymerase [35], cut by KpnI and EagI and, finally, 1.9 ng of double digested filled duplexes was ligated to 200 ng of low-melting point agarose gel-purified large fragment of the replicative form (RF) Ph.D.121 DNA cut with the same pair of enzymes (the Ph.D.121 was a randomly picked phage clone from the Ph.D.-12 phage display peptide library) in a total volume 0.1 ml. Ligations were electroporated into 0.5 ml of TG-1 electrocompetent cells (Stratagene) according to manufacturer's protocol and phage library complexities were determined by immediate mixing of several tenfold dilutions of transfected TG-1 cells with ER2537 cells and plating. Phage libraries were further amplified in 400 ml of liquid LB media for 4 h at 37°C with vigorous shaking, concentrated by PEG (see above) and, finally, suspended in 0.5 ml TBS + 0.05% sodium azide buffer. Titers of four resultant nonrandom combinatorial phage libraries were $0.2–4.0 \times 10^{13}$ pfu/ml. For a long-term storage phage libraries were adjusted to 50% glycerol and stored at -20°C. Twelve independent phage clones

from each TR401-91CL and RhR401-91CL libraries were sequenced in order to determine the average level of amino acid substitutions in the fluorette moiety.

Biopanning with mutagenized combinatorial phage display peptide libraries.

The libraries were selected against the corresponding fluorophore dye carrier. All biopanning rounds were performed essentially as for biopanning with the Ph.D.-12 phage display peptide library, round 1 (see above) except input phage in the binding reaction were decreased to 1.0×10^{10} pfu, time of binding was reduced to 10 min, amount of fluorophore dye carrier beads in the binding reaction was reduced to 0.03 ml, following binding to the phage beads were washed with 330-fold beads volume of washing buffer and Tween-20 concentration in the washing buffer was increased to 0.4%.

Phage–fluorophore-dye binding in solution

Four activated derivatives of fluorophore dyes (see above) were quenched in 0.5 M Tris–HCl, pH 8.7 and used as 'free' dyes. Ten µl of PEG-purified and 300-fold concentrated phage TR401, RhR401, OG402, OG403 ($1.3–1.8 \times 10^{12}$ phage particles; calculated from optical densities of the purified phage at 260 and 280 nm) in TBS + 0.05% sodium azide buffer were separately mixed with an equal volume of 20 µM solution of the corresponding free dyes in the same buffer; mixtures were adjusted by Tween-20 to 0.1% and incubated for 3 h. Phage-fluorophore dye complexes were precipitated three times by PEG in order to remove unbound dye and dissolved in 6.6 µl of TBS + 0.05% sodium azide buffer. Finally, phage-fluorophore dye complexes (1 µl of each) were spotted to nitrocellulose filter and filter was scanned on the Storm 840 scanner (Molecular Dynamics) in Blue Fluorescence/Chemifluorescence mode with 200 µ pixel resolution. To determine nonspecific binding the normalized amounts of phage particles of amplified and PEG-purified Ph.D.-12 phage display peptide library were separately incubated with the corresponding free dyes and treated in the same conditions. All binding experiments were accomplished in duplicate. Specific/nonspecific signal ratios were quantified by densitometry of spot images using 'Measure' option of NIH Image 1.59 freeware package.

Phage dissociation constant measurements

PEG-purified TR401 phage, TRS311 phage or the amplified Ph.D.-12 phage display peptide library phage as a negative control (input $1.5 \times 10^8–1.1 \times 10^{10}$ pfu with twofold to fivefold increments) were incubated with 23.5 µl of BSA-blocked and washed Texas red carrier beads in binding buffer (see above) in total volume 40 µl for 3 h. Beads suspensions were centrifuged, the supernatants (unbound phage) were titrated and dissociation constants were measured via a standard linear Scatchard plot. For TR401 and TRS311 phage bound/unbound ratios were quite reliable (6.3–13.1 in the range of phage concentrations shown above). All binding experiments were accomplished in duplicate and titrations were performed in triplicate. This direct binding assay can reliably measure K_d if it does not exceed ≈1.5-2 nM. For higher K_d, bound/unbound ratios became unreliable (≤1).

Other PEG-purified phage (input $5 \times 10^8–2.5 \times 10^{10}$ pfu with 2 to 5-fold increments) were bound to the respective fluorophore dye carriers essentially in the same manner as described above for Texas red-specific phage. RhR401 and RhRS308, OG403 and OGS316, OG402 and FluS303 phage, were bound to the Rhodamine red, Oregon green 514 or fluorescein carriers, respectively. Following binding the beads were quickly washed twice by 0.25 ml of TBS + 0.1% Tween-20 and suspended in 10 ml TBS. 10 µl of suspension was mixed with ≈2×10^8 log-phase ER2537 cells and incubated 1 h at 4°C with slight shaking to allow phage adsorption. Several ten-fold dilutions of infected cells were mixed with noninfected ER2537 cells and plated in standard plaque assay [34]. All binding experiments and titrations were accomplished in duplicate. Nonspecific background of binding (determined with amplified Ph.D.-12 phage display peptide library phage) did not exceed more than 3% of specific

binding in each case. For all above phage pfu/particle ratio was in the range 0.4–0.5.

Peptide synthesis and concentration measurement
Eight 29-mer peptides (see Table 4) were synthesized by American Peptide Company and HPLC purified. Peptide purity was 95–99%. Correct molecular weights of peptides were confirmed by mass-spectrometry. Peptides were dissolved in TBS buffer and concentrations were determined by amino acid analysis of alanine and glycine content.

Peptide–Texas-red binding
Bead binding. The same amounts of Texas red-specific peptides TR401, TRS311, TR406 as well as nonspecific peptide control (see Table 4) were bound via His$_6$ tag to the TALON Metal Affinity Resin (Clontech) in TBS buffer essentially as described by manufacturer's protocol. Beads (5 µl) were washed twice with 0.5 ml TBS to remove unbound peptides and incubated with 0.5 µM Texas red in 40 µl of TBS buffer for 1 h at RT. Finally, the beads were washed three times with 0.3 ml TBS to remove unbound dye. Fluorescent and nonfluorescent control beads were photographed with 10-fold magnification on fluorescent microscope Axiophot (Zeiss) using Rhodamine red/Texas red filter with 5 sec exposure .

Binding of fluorette peptides to dyes in solution. Peptides in different concentrations (1.3–20 µM) were incubated with Texas red (50 nM) in 0.6 ml of TBS buffer for 1 h at RT. Excitation and emission spectra of samples were obtained by using spectrofluorimeter SPEX Fluoromax (Jobin Yvon-SPEX Instruments Co.) and DataMax software package. Peak positions were automatically calculated by software. List mode data was saved for other analyses.

Peptide–Texas-red dissociation constant measurements
Equal amounts of Texas red-specific peptides TR401 and TRS311 as well as nonspecific peptide control (Table 4) were bound to the TALON Metal Affinity Resin (Clontech) in TBS buffer essentially as described by manufacturer's protocol. Beads loaded with peptides (160 µl) were washed three times with 1 ml of TBS buffer to remove unbound peptides and, finally, 10 µl of washed beads was incubated with three-fold increment of concentrations of Texas red in 40 µl of TBS buffer for 1h at RT. Beads were placed in microcolumns and quickly washed with 0.1 ml TBS to remove unbound dye. Bound peptide - Texas red complexes were then eluted by 0.24 ml of 50 mM sodium phosphate, pH 5.7 plus 100 mM NaCl. The volume of eluates was adjusted to 0.625 ml by an addition of 150 mM Tris-HCl, pH7.5 and concentrations of Texas red in samples were measured using spectrofluorimeter SPEX Fluoromax (Jobin Yvon-SPEX Instruments Co.) with known Texas red concentrations as standards (excitation at 589 nm and emission readout at 605 nm). Nonspecific binding of Texas red (binding to the beads loaded with peptide control did not exceed 0.8% of the specific binding) was subtracted. Scatchard plots for TR401 and TRS311 were linear in the range of 0.01–0.71 µM and 0.008–1.09 µM bound Texas red, respectively (see Figure 6). All binding experiments as well as Texas red concentration measurements were accomplished in duplicate.

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