Chemistry & Biology, Vol. 11, 347–356, March, 2004, ©2004 Elsevier Science Ltd. All rights reserved. DOI 10.1016/j.chembiol.2004.03.004

# In Vivo Targeting of Organic Calcium Sensors via Genetically Selected Peptides

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## Summary

**A library of constrained peptides that form stable folded structures was screened for aptamers that bind with high affinity to the fluorescent dye Texas red. Two selected clones had binding constants to Texas red of 25 and 80 pM as phage and binding had minimal effects on the fluorescence of Texas red. The peptides interact with distinct but overlapping regions of Texas red. One peptide bound to X-rhod calcium sensors, which share the same core fluorophore as Texas red. These dyes retained calcium sensitivity when bound to the peptide. This peptide was used to label a fusion protein with X-rhod-5F in vivo, and X-rhod sensed changes in calcium locally. Thus, minimal, constrained peptides can functionally bind to environmentally sensitive dyes or other organic agents in biological contexts, suggesting tools for in vivo imaging and analysis.**

## Introduction

It is increasingly clear that the specificity of cell signaling can be encoded by the spatial and temporal distribution of signaling proteins and second messengers, such as nitric oxide [1], cAMP [2], zinc [3], and calcium [4]. For example, in B cells, differential activation of transcription factors is observed when the frequency, duration, or amplitude of calcium oscillations is changed [5]. In addition, localized changes in intracellular calcium regulate processes including muscle contraction, hormone secretion, transcription, and neurotransmitter release [6]. Thus, mechanistic understanding of calcium-mediated signaling requires assays that can resolve the spatial and temporal pattern of signaling events. Since many signaling pathways interact with calcium-mediated signals, the network properties of mammalian signaling can only be evaluated with simultaneous measure of multiple signaling nodes in single cells [7]. Fluorescence assays are well suited to such investigations since they can generate highly specific signals that can be detected in single, living cells.

While GFP and related proteins are now widely used to report protein localization, they have proven difficult to use as sensors of critical biochemical changes such as second messenger concentration, protein phosphorylation, and protein-protein interactions [8]. Fluorescent protein sensors of $Ca^{2+}$ have been created, but the use of these fluorescent proteins is hampered by size and the inherent insensitivity of the fluorophore—the changes in fluorescent signal are typically quite small and can require complex experimental design to measure signal above background [9–11]. Organic fluorescent dyes can have far more sensitivity to their environment than has been achieved with fluorescent proteins, despite broad efforts to modulate the environmental sensitivity of fluorescent proteins [12]. For example, there are several classes of organic calcium-sensitive fluorescent dyes that can undergo at least a 100-fold increase in fluorescence in the presence of calcium [13]. In this work, we extend the abilities of a platform that allows labeling of intracellular proteins with small molecule dyes, putting the sensitivity of organic dyes under genetic control. Unlike fluorescent proteins, fluorescent small molecules are available in a wide range of spectral properties, including a large family of dyes that can measure intracellular calcium concentrations.

Short, dye binding peptides are an approach that might combine the versatility of small molecule fluorescent dyes with the genetic tractability of fluorescent proteins. A tetra-cysteine motif has been elegantly used to interact with, and thus target, arsenic-containing fluorescent dyes, but background staining and toxicity have prevented widespread use [14, 15]. In previous work, we used phage display to identify a 13-mer peptide that noncovalently binds with 1 μM Kd to the fluorescent dye Texas red [16]. These peptides proved the potential to create genetic control of the targeting of small molecules. However, though this was a proof of concept, the affinity of the linear peptide for the fluorophore was too modest to be generally useful in biological settings. We therefore sought to create higher-affinity, versatile peptide structures that allowed design-dependent localization of organic small-molecule calcium sensors.

To achieve high-affinity interactions, we developed a phage library that uses a short, 7 amino acid dimerization domain to constrain peptides [17]. This sequence is one of the smallest known domains for peptide libraries that is stable across a broad range of physiologic conditions [18]. We show here that this library yields high-affinity peptides that bind to Texas red and calcium-sensitive X-rhod dyes. The selected peptides bind with sufficient affinity to use the peptide as a tag or tracer in vitro and in vivo.

## Results

### Library Creation and Selection of Texas Red Binding Phage

Two peptide libraries were created with randomized sequences flanked by the SKVILFE dimerization domain (Figure 1A). This domain has been shown to provide conformational constraint and added stability against protease action to the selected peptide [18]. Libraries of two lengths were created and screened to test for bias based on the insert length. A 9-mer library with

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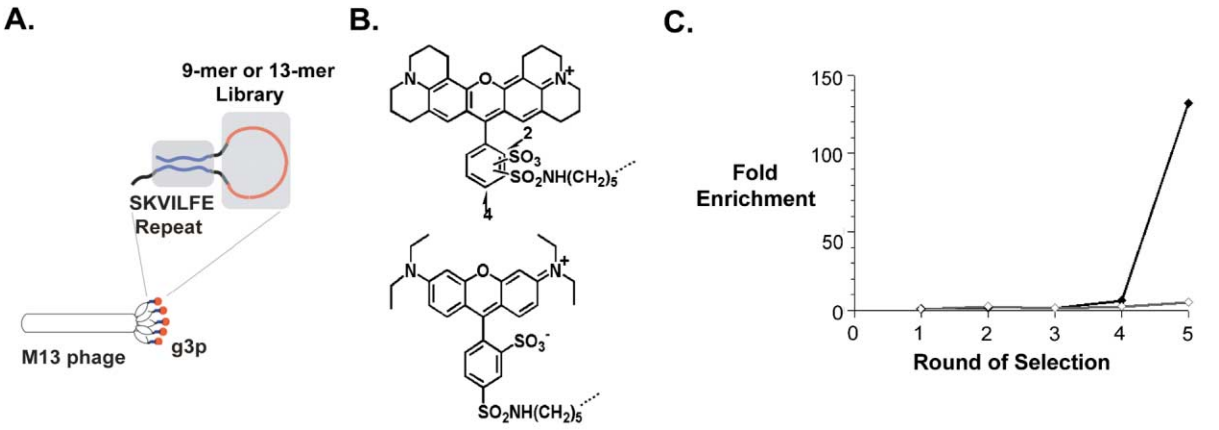


D.

| clone | frequency | | insert sequence | | Kd (phage) |
|---|---|---|---|---|---|
| tr501 | 41/48 | GGG SKVILFE GPAG | RTIWEPKEASNHT | GAPG SKVILFE GGPG | 80 pM |
| tr512 | 1/48 | GGG SKVILFE GPAG | RWTWPEISE | GAPG SKVILFE GGPG | 25 pM |
| tr505 | 1/48 | GGG SKVILFE GPAG | WSKMGHTVT | GAPG SKVILFE GGPG | none detected |
| tr518 | 1/48 | GGG SKVILFE GPAG | GNQKCLQHNRQST | GAPG SKVILFE GGPG | none detected |
| tr526 | 1/48 | GGG SKVILFE GPAG | SQTWSFPEH | GAPG SKVILFE GGPG | none detected |
| tr527 | 1/48 | GGG SKVILFE GPAG | EPMARPWERKQDR | GAPG SKVILFE GGPG | none detected |
| tr541 | 1/48 | GGG SKVILFE GPAG | GTISATRPYGRQW | GAPG SKVILFE GGPG | none detected |

Figure 1. Selection of Texas Red Binding Phage from a New Constrained Library

(A) 9-mer and 13-mer peptide libraries were created and flanked on each side by the homodimerization domain with sequence SKVILFE.

(B) Texas red (top) and rhodamine red (bottom) were used as target molecules in phage display screening. In both molecules, the positive charge can be shared over the $\pi$-bond system and one major resonance form is shown here. Dashed lines represent the point of conjugation to the solid support.

(C) Strong enrichment of Texas red selected phage over five rounds of selection measured by the ratio of eluted phage/input phage for each round (Texas red, closed circles; rhodamine red, open circles).

(D) Analysis of individual phage clones present after round 5 of screening against Texas red. Phage clones were sequenced and the binding constant (Kd) of each clone for Texas red was determined using Scatchard analysis.

$3.1 \times 10^8$ different inserts and a 13-mer library with $1.25 \times 10^8$ different inserts were pooled and used in these studies. To determine the ability of these constrained libraries to yield useful, high-affinity peptide binders, phage display selections were performed using Texas red and rhodamine red as the target antigens. We previously used these antigens in screens with a linear peptide library. This permits comparison of the affinities and properties of the peptides both in the context of the selected phage and as free peptides.

Texas red and rhodamine red were conjugated to a solid support by reaction of the succinimidyl ester of each dye with a Diaminodipropylamine (DADPA) column that contains free amine groups for coupling (Figure 1B). This forms a stable amide bond and contains a 19-atom linker that minimizes possible steric hinderance by the column. Five successive rounds of biopanning were performed in parallel for Texas red and rhodamine red binding phage (Figure 1C). After 5 rounds of selection, there was a 140-fold enrichment in phage that bound to Texas red, but only a 5-fold enrichment in rhodamine red binding phage. We focused on the characterization of potential Texas red binding aptamers, since known derivatives of Texas red with interesting optical and chemical properties (see below) could provide further tests of the potentials of the peptide-fluorophore interactions.

### Analysis of Phage Clones

Forty-eight Texas red binding clones were isolated and sequenced. One clone, TR501, represented 85% of the phage present after five rounds of selection. Seven other phage clones were each present once out of the 48 sequenced clones (Figure 1D). Eighteen clones from the unselected library were sequenced and compared to the Texas red-selected phage. The selected phage had a different amino acid composition compared to the unselected clone group: we noted that hydrophobic residues were selected against, while acidic and basic residues were greatly enriched (data not shown). The eight phage clones identified after five rounds of screening against Texas red were each tested for binding to Texas red. Only TR501 and TR512, which share a sequence similarity (RxxWEP), bound measurably to the Texas red column, without background binding to a control column lacking Texas red. Binding constants for those interactions were determined by Scatchard analysis (Figure

1D). These two phage clones bound to Texas red with binding constants of 80 and 25 pM, respectively. These apparent binding constants might reflect avidity in the interaction between the phage and Texas red, as five copies of the peptide are present on each phage particle. Thus, phage binding constants are best applied to measure the relative binding of a series of selected peptides to a single antigen. In this case, TR501 and TR512 bind 3.4- and 10.8-fold better than the phage clone that was previously isolated from a linear, 12 amino acid library [16]. Thus, the SKVILFE-constrained peptides can be a source of higher-affinity agents than the linear peptides previously selected.

### In Vitro Binding Assays with Purified Peptides

It has been previously observed that phage display-selected aptamers often have avidity for targets that is dependent on local phage context [19]. If the aptamers are removed from the phage, they lose their affinity for the target. For example, with a linear peptide library screen, only 2 of 8 peptides bound to target fluorophores when taken out of the context of the phage [16]. We hypothesized that the SKVILFE constraint would minimize this steric dependence since it could fold independently into a stable domain and may not require adjacent phage sequences to provide constraint or scaffolding for binding. To test this, and to evaluate uses of the Texas red binding sequences in vitro, we synthesized $His_6$-tagged versions of the selected peptides. These peptides were bound to cobalt beads for in vitro binding assays via microscopy and flow cytometry. Bead bound peptides were incubated with Texas red and washed, and the retained fluorescence was assessed by flow cytometry (Figure 2A) and microscopy (Figures 2B–2E). TR401 is a linear peptide with the sequence KHVQYWT QMFYS, which was isolated previously, and binds to Texas red with a binding constant (Kd) of 1.6 $\mu$M [16]. Both SKVILFE-constrained peptides TR501 and TR512 bind tightly to Texas red. The flow cytometric assay reveals that TR512-loaded beads have 300-fold more Texas red fluorescence intensity, compared to a SKVILFE-constrained control peptide. In vitro, pure TR512 peptide retains 100-fold more Texas red than TR401, the peptide generated in a screen of a linear library. In this assay, free dye is lost when the beads are washed, and therefore Texas red binds to peptide TR501 and TR512 with sufficient affinity to maintain the interaction over the span of several washes and the length of the assay ($>$30 min).

The Texas red-peptide interactions were readily detected by microscopy (Figures 2B–2E). Control beads, to which a nonspecific SKVILFE-constrained peptide had been linked via $His_6$ tag, had no detectable fluorescence, while the SKVILFE-constrained peptides TR501 and TR512 clearly bind to Texas red. These experiments recapitulate the flow cytometric assay of binding, in that TR512 binds better to Texas red than TR501, but both bind Texas red far better than a control peptide or the linear peptides we previously selected. Thus, these SKVILFE-constrained peptides retain binding to Texas red when removed from the phage context. Notably, these experiments reveal that the peptide did not grossly quench the fluorescence of Texas red upon binding. In fact, incubation of 20-fold excess peptide with TR501 or TR512 caused a 30% increase in the intensity of red fluorescence compared to dye alone (Figures 2F and 2G). In addition, peptide binding to Texas red causes a minimal shift in its excitation and emission properties, as each peptide causes a 2 nm shift in excitation and emission (Figures 2F and 2G). Interestingly, a similar shift was seen previously with the linear TR binding peptides [16].

To clarify the sequence and structural requirements for binding to Texas red-related dyes, Glutathione-S Transferase (GST) fusions of the peptides and several variants were generated and purified. To test whether the SKVILFE constraint was required for binding, we created variants where the SKVILFE regions were (1) removed completely, creating a linear peptide, or (2) replaced with cysteines, which can form a constraint via disulfide bridge. Removal of the SKVILFE regions from TR512 or TR501 greatly reduces binding to Texas red (Figure 3A). This binding is further reduced in the linear version of the peptides, suggesting strongly that some form of structural constraint is required for binding. It is also possible that the SKVILFE participate in some contacts with the Texas red fluorophore.

Texas red's structure consists of a core xanthene fluorochrome consisting of three fused rings, plus four outer conjugated rings that modify the fluorescence of the xanthene core. To test whether this entire series of conjugated rings was required, we tested binding to rhodamine red, a related molecule that lacks the outer fused rings (Figure 3B). TR512 still binds effectively to rhodamine red, while TR501 does not. This suggests that TR512 binds to the central three fused rings, while TR501 binds to a larger surface area of Texas red. We tested several Texas red derivatives that contain different groups on the aromatic ring opposite the fluorochrome region (Figure 3B). This region of Texas red was coupled to the solid support in the initial selection, and in X-rhod dyes, this region contains a calcium chelating group that makes the red fluorescence sensitive to calcium. TR512 bound effectively to all of the derivates, demonstrating that TR512 requires only the central xanthene core of Texas red/rhodamine red for binding. Two conservative views of the derived interaction surface are shown in Figures 3C and 3D. Figures 3C–3F summarize the structural requirements of Texas red for binding to the peptides. An energy minimization model of Texas red-succinimidyl ester is presented; alteration or removal of the regions that are highlighted in yellow caused loss of binding to the peptide. Regions that could be altered without affecting binding to the peptide were determined to be outside of the binding surface. Specifically, there does not appear to be a need for the outer conjugated ring elements to be maintained in their fused ring form, and the nitrogens could still contribute to binding. TR501, in contrast, exhibited variable binding to the derivatives. In particular, TR501 did not bind to Texas red cadaverine, which contains an amine group at the end of a 5-atom hydrocarbon chain. The inhibitory effect of the amine group suggests that this region of Texas red is required for binding to TR501 or that the amine group can disrupt charge-charge interactions that are important for binding. Both alternatives impli-

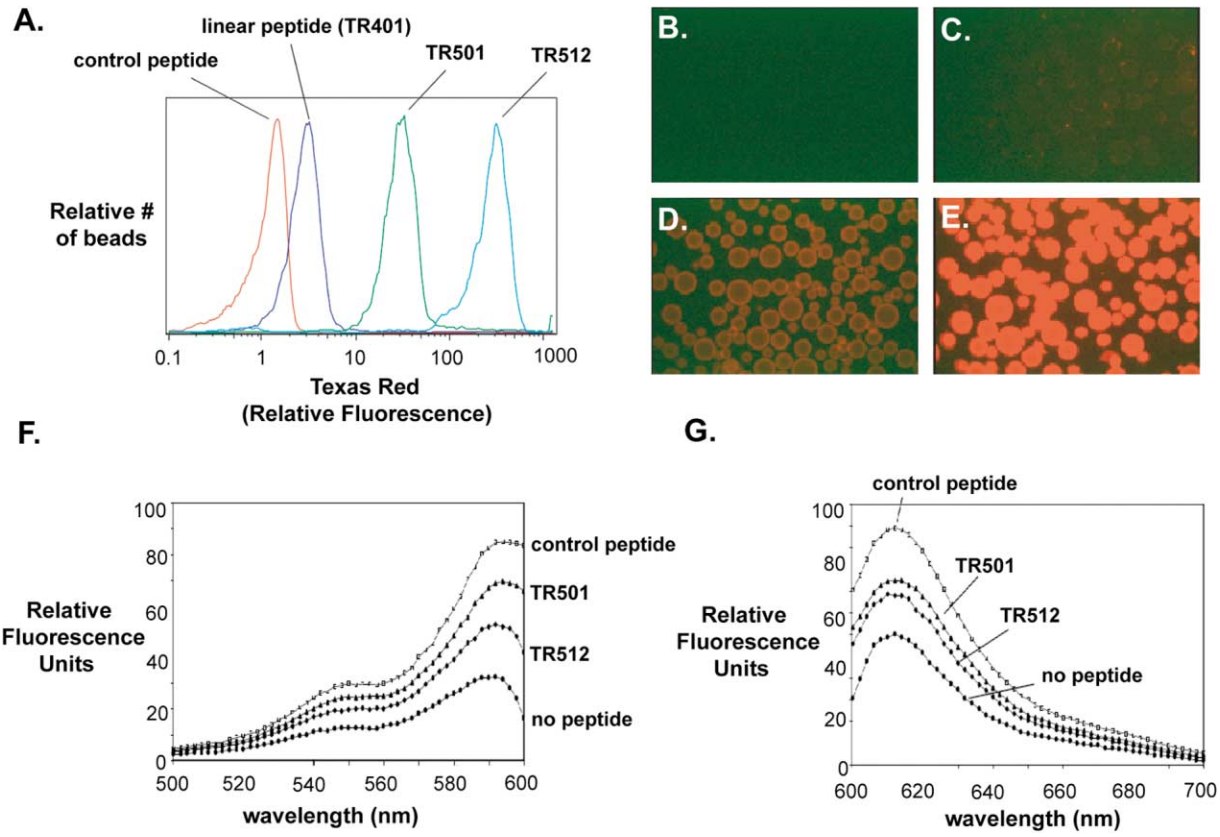


Figure 2. TR501 and TR512 Bind Effectively to Texas Red

(A) Nickel beads were loaded with synthetic $His_6$-tagged peptides and allowed to bind to Texas red. Relative Texas red fluorescence per bead was then measured by flow cytometry using standard settings [36]. The histograms indicate the Texas red fluorescence of 20,000 beads for each sample for SKVILFE-constrained control peptide (red histogram) a peptide selected against Texas red from a linear library, TR401 (dark blue), and constrained peptides TR501 (green) and TR512 (light blue).

(B–E) Microscopic analysis of binding to Texas red. A SKVILFE-constrained control peptide (B), TR401, a peptide selected against Texas red from a linear library (C), and peptides TR501 (D) and TR512 (E) were bound to beads and incubated with Texas red. Images were collected identically for all samples.

(F and G) Excitation and emission spectra of Texas red incubated with 10-fold excess peptide.

cate this region of Texas red as capable of contributing in the binding to TR501. The potential interaction surfaces for TR501 and Texas red are shown in Figures 3E and 3F.

### In Vitro Binding to X-Rhod Calcium Sensors

X-rhod dyes contain the Texas red fluorophore and a BAPTA calcium chelator (Figure 4A). The dyes are largely nonfluorescent, unless bound by calcium, when they undergo a 100-fold increase in fluorescence. Because X-rhod dyes share the regions of Texas red that were required for TR512 binding, we hypothesized that TR512 could bind to X-rhod dyes and that binding to TR512 may not interfere with X-rhod binding to calcium. To test this, we incubated various X-rhod dyes with TR512 peptide-loaded beads (Figure 4B). All four X-rhod dyes bound to TR512 and were only fluorescent in the presence of calcium. Very little fluorescence was detected upon X-rhod binding to TR501-loaded beads, suggesting that the interaction is not as strong or that the binding interferes with the calcium-sensitive fluorescence of X-rhod-1 (data not shown). To test the calcium sensitivity of the bound X-rhod dyes, the emission spectra of X-rhod-1 was analyzed with 10-fold excess of TR512 or a control peptide, in the presence of 100 mM $Ca^{2+}$ or without added calcium but with 100 mM EGTA to chelate any possible free calcium (Figure 4C). All four X-rhod dyes that were tested underwent a dramatic calcium-dependent increase in fluorescence in the presence of 10-fold excess TR512 peptide. Binding to TR512 did not cause any detectable differences in the calcium sensitivity of X-rhod, as calcium response curves were identical in the presence and absence of excess peptide (data not shown). The substitutions on the BAPTA chelator that define the various X-rhod dyes are known to cause large differences in the calcium sensitivity of the dye. The range of calcium concentrations that are sensed by TR512 bound X-rhod dyes was tested in vitro by incubating 10-fold excess peptide with each of the dyes in the presence of a wide range of calcium (Figure 4D). The results show that the four X-rhod dyes together can measure calcium over a 1000-fold range, from 100 nM to 100 μM or more, when bound individually to TR512 peptide.

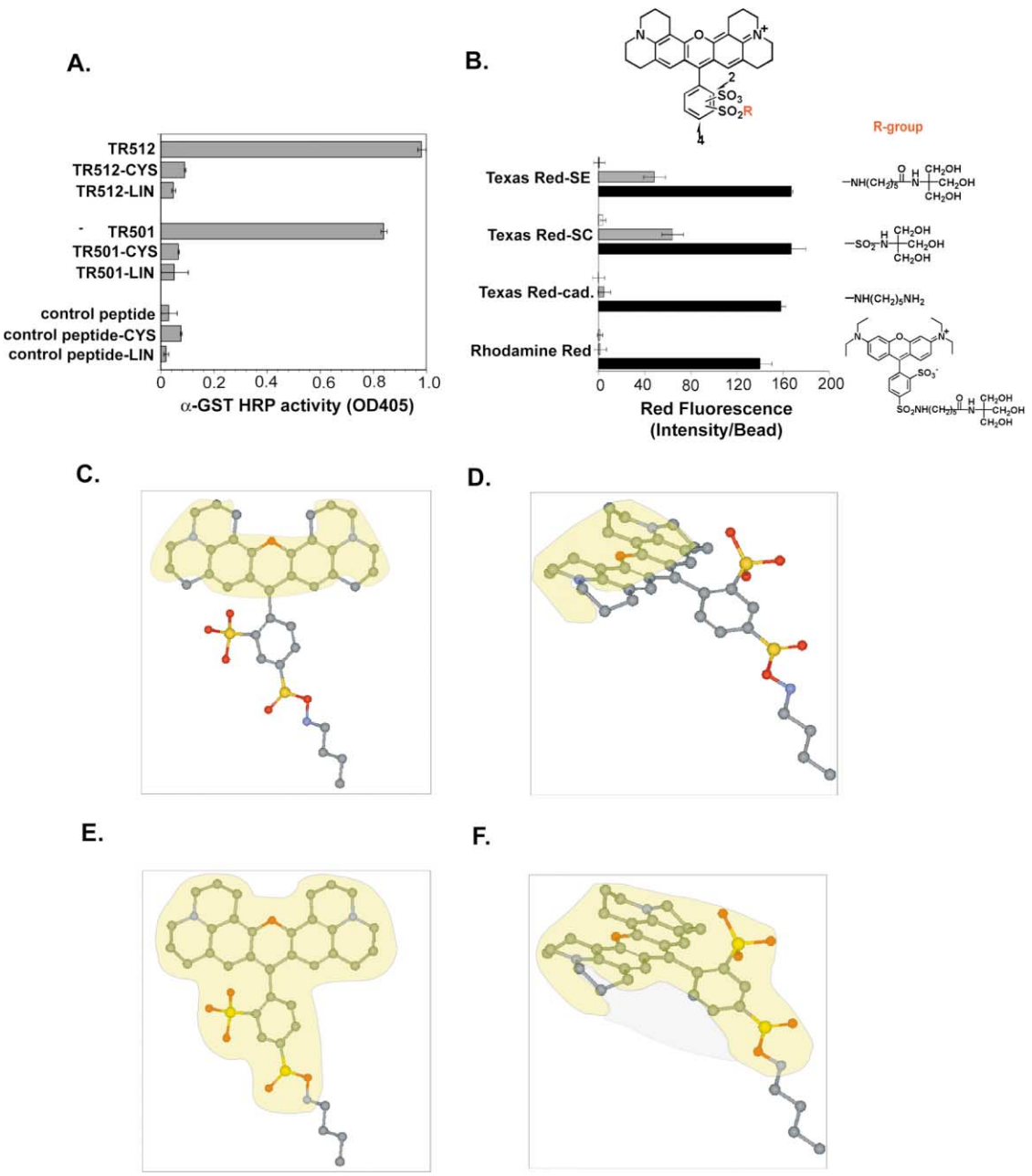


Figure 3. SKVILFE Dimerization Sequences Are Required for High-Affinity Binding to a Range of Texas Red Molecules

(A) ELISA assay of GST fusion peptide binding to Texas red-BSA coated plates, detected with HRP-anti-GST antibody.

(B) Peptide-dye binding was assayed for several Texas red derivatives following incubation of $His_6$-tagged peptide and a Texas red derivative and purification of the complex with cobalt beads. As previously, one resonance form of Texas red is shown.

(C and D) Energy minimizations of Texas red (succinimidyl ester) with the region required for binding to peptide TR512 highlighted in yellow.

(E and F) Energy minimizations of Texas red with the potential interaction surface for TR501 highlighted in yellow.

### Texas Red Binding Peptides Localize Calcium-Sensing X-Rhod Fluorophores

We tested the utility of the interaction to label proteins inside of living cells. The peptides were expressed as fusion proteins in NIH3T3 cells, and confocal microscopy was used to assess X-rhod-5F labeling of the fusion protein. Retroviral constructs were used to express TR512 or the control peptide as a C-terminal fusion to a membrane-targeted GFP construct. Membrane targeting was achieved via an N-terminal, 10 amino acid signal sequence from the Src family kinase Lyn. Stable cell lines were created and shown by FACS to express similar levels of Lyn-GFP-peptide (data not shown).

Control NIH3T3- or Lyn-GFP-TR512-expressing NIH3T3 cells were incubated with the cell-permeable, acetoxymethyl-ester form of X-rhod-5F, and the localization of GFP and red fluorescence was examined by confocal microscopy (Figure 5). To test the calcium sensitivity of

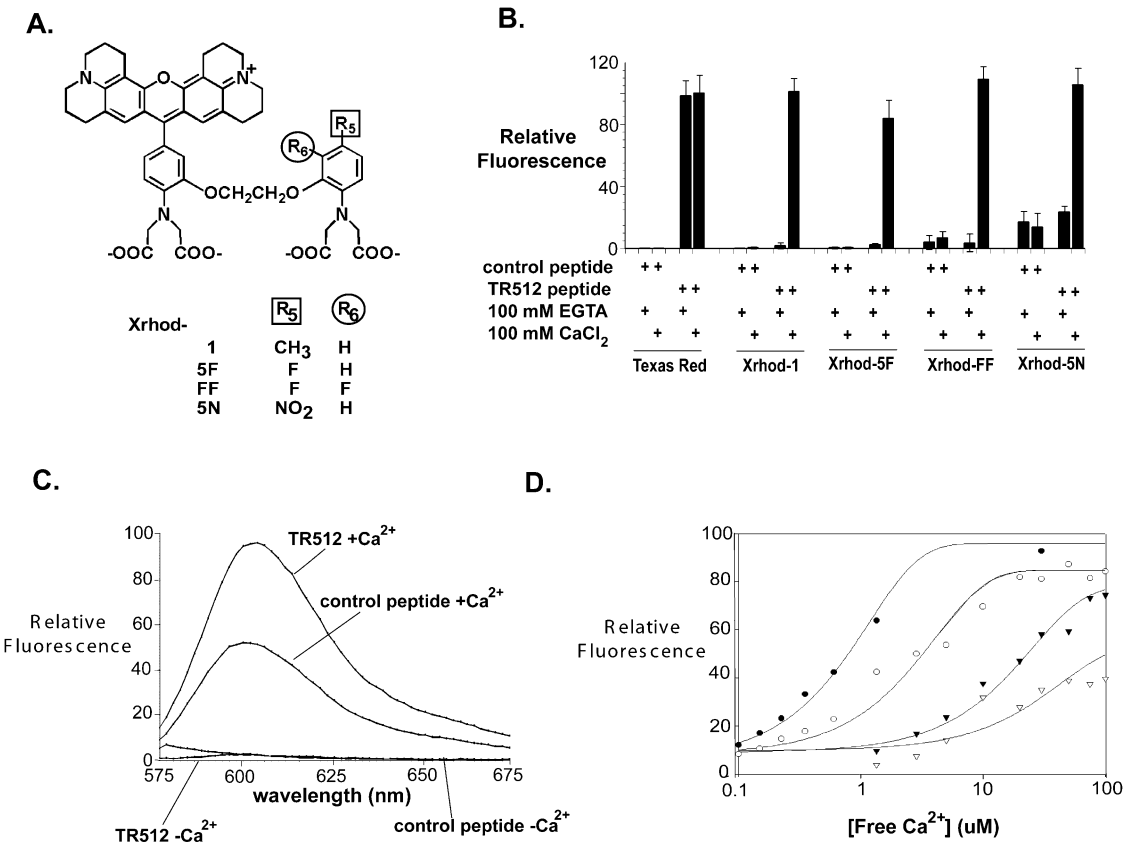


Figure 4. TR512 Binds to Calcium-Sensitive X-Rhod Dyes

(A) The generic structure of the X-rhod dyes (Texas red derivatives with a BAPTA chelator) is shown. Substitutions at R groups are shown for the X-rhod derivatives that were analyzed. Like Texas red, the positive charge can be shared over the $\pi$-bond system and one resonance form is shown here.

(B) Red fluorescence of X-rhod dyes associated with $His_6$-tagged peptide loaded beads in the presence and absence of $Ca^{2+}$.

(C) Fluorescence emission spectra of X-rhod-1 with 10-fold excess of control peptide or peptide TR512.

(D) $Ca^{2+}$ sensitivity of X-rhod-1 (filled circles), X-rhod-5F (open circles), X-rhod-FF (closed triangles), and X-rhod-5N (open triangles) after binding with 10-fold excess TR512.

the X-rhod-5F, cells were treated with ionomycin. As expected, X-rhod-5F fluorescence levels in cells increased upon addition of ionomycin, which has been shown to increase intracellular $Ca^{2+}$ levels from a resting level near 100 nM to 1–5 $\mu$M [9]. Control NIH3T3 treated with ionomycin exhibited cytoplasmic X-rhod-5F staining, with some punctate staining and no detectable green fluorescence (Figures 5A and 5B). Meanwhile, ionomycin-treated cells expressing membrane-targeted TR512 had clear plasma membrane X-rhod-5F staining (Figure 5C). The X-rhod-5F staining colocalized with green fluorescence from the lyn-GFP-TR512 protein, indicating that X-rhod-5F is targeted to TR512 in vivo (Figure 5D). Unstained Lyn-GFP-TR512 cells had no detectable red fluorescence, indicating that there were no long wave emissions from GFP into the red fluorescence of X-rhod-5F (Figures 5E and 5F). Quantitation of the relative fluorescence at the plasma membrane and the rest of the cell revealed that 71.1% of X-rhod-5F fluorescence was localized to the plasma membrane in the cell expressing membrane-targeted Lyn-GFP-TR512 (Figure 5C). In comparison, 85.3% of the GFP fluorescence of Lyn-GFP-TR512 was localized to the plasma membrane (Figure 5D). This suggests that a portion of the X-rhod-5F background is in fact X-rhod-5F labeling of Lyn-GFP-TR512 molecules that have not been lipid modified and targeted to the plasma membrane. In a cell lacking Lyn-GFP-TR512 (Figure 5A), only 7.0% of X-rhod-5F fluorescence was localized to the plasma membrane. Thus, TR512 causes qualitative and quantitative targeting of X-rhod-5F in vivo.

## Discussion

### Creation of a New Peptide Library Scaffold

A goal of this work was to further develop a system by which one can flexibly label proteins and other intracellular molecules with fluorescent dyes inside of living cells. To do this, we developed an improved peptide library system. Linear peptide libraries typically yield lower affinity interactions, and those interactions appear to be very sensitive to the local environment in which the peptide was selected [20, 21]. In this work, we compared the relative Texas red binding of a linear peptide that we previously isolated to the peptides that we isolated from the new constrained library. As phage, the

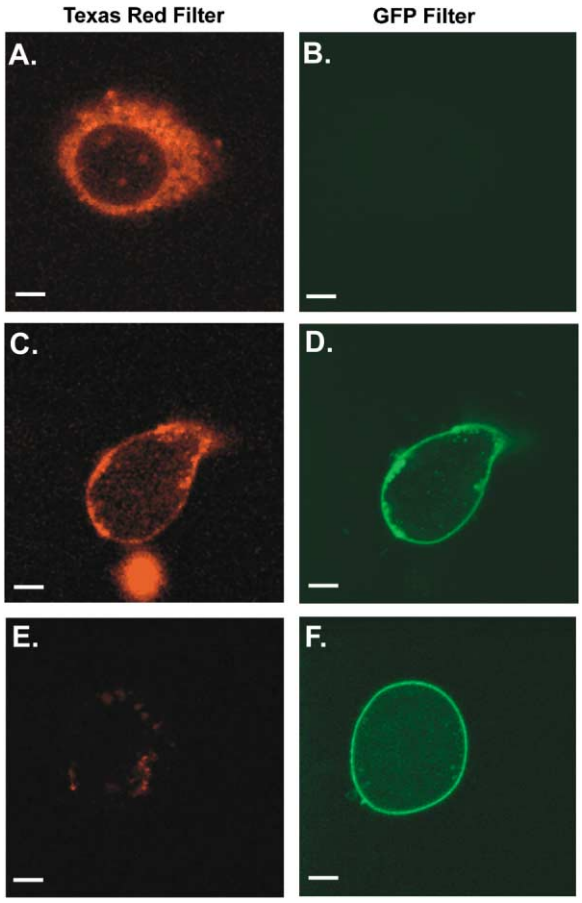


Figure 5. X-Rhod-5F Selectively Labels TR512 Fusion Proteins in NIH3T3 Cells

Confocal microscope images of control NIH3T3 and NIH3T3 that express lyn-GFP-TR512. Cells were simultaneously imaged for Texas red (A, C, and E) and GFP (B, D, and F).

(A and B) Control NIH3T3 treated with X-rhod-5F-am and ionomycin.

(C and D) Lyn-GFP-TR512 expressing NIH3T3 treated with X-rhod-5F-am and ionomycin.

(E and F) Lyn-GFP-TR512 expressing NIH3T3 treated with ionomycin but not X-rhod-5F-am. Scale bars equal 5 $\mu$m.

constrained clones bound 3.4- and 10.8-fold better than the linear clone. A similar trend was observed in in vitro binding assays, where the constrained peptides retained Texas red much better than the linear peptide. Future work to identify the $K_{on}$ and $K_{off}$ of these interactions in solution might provide insights into the improved interaction with Texas red. Cysteines have been widely used to constrain peptide libraries [22]. While permitting a very small overall size of the recognition domain (the library plus two cysteines, at a minimum), the constraint depends on formation of the disulfide bridge [23]. Therefore, cysteine-flanked libraries are not perforce constrained when present in a reducing environment (like the cytoplasm where the disulfide bridge is unlikely to form) compared to oxidizing environments (i.e., extracellular or secretory where the disulfide bridge forms).

Both TR501 and TR512 retained binding when the peptide was removed from the N terminus of the phage coat protein and expressed as a C-terminal GST fusion. This portability has held true in two other screens for peptides that bind to other small molecules (K.M.M. and G.P.N., unpublished results). This demonstrated that SKVILFE-flanked libraries yield stable microdomains that can retain activity across multiple settings. This could be due to the increased stability and structure of the SKVILFE-flanked sequences or due to higher affinity of peptides selected from the SKVILFE library. Such a result could hold true for other microdomains of differing structures. In our previous work with linear peptide libraries selected for binding to fluorescent dyes, only 2 out of 8 selected peptides retained detectable binding when removed from the phage [16].

### Characterization of Texas Red Binding Peptides

The two phage clones that bind to Texas red share a conserved motif that is absent from the nonbinding clones that remained following the selection. Both phage clones contain the motif RxxWEP, starting at the first amino acid of the randomized region. The probability that two phages from the library would both randomly contain this motif is 1 in $3 \times 10^{11}$. Thus, its presence in both binding phage implicates these residues as key portions of the binding region of these peptides. It is possible that the arginine and glutamate residues present in both TR501 and TR512 are spaced appropriately to engage in charge-charge interactions with the exocyclic nitrogen of Texas red. This is an intriguing possibility, as X-ray crystallography revealed that the anti-fluorescein antibody 4-4-20 interacts with fluorescein via a salt bridge and a hydrogen bond to the two enolic groups on fluorescein that occupy the identical position as the exocyclic nitrogens of Texas red [24]. Such a conclusion for the Texas red binding peptides awaits structural characterization by NMR or other techniques. Interestingly, the RxxWEP motif begins at the same point relative to the SKVILFE dimerization domain in both clones; this suggests that the orientation of the motif is important for binding or that the actual binding surface includes part of the SKVILFE scaffold. Because the scaffold contains a hydrophobic core (with the amino acid sequence VILF), it may be able to stabilize the interaction between peptide and dye via interactions with the hydrophobic regions of the polyaromatic core of Texas red. In this work, three fluorescent dyes were tested as antigens for phage display. Texas red yielded the clearest enrichment (140-fold) and was characterized in depth. A more modest 5-fold enrichment was observed for rhodamine red binding phage. Oregon green was also tested, and under these conditions, enrichment for Oregon green binding phage was not observed. Texas red and rhodamine red are both charged at physiologic pH and might therefore present a more complex binding surface than Oregon green. It is also possible that methodological changes during the selection would permit selection of Oregon green binding phage, as only one selection protocol was attempted. It is likely that this library can yield peptides that interact with a wide range of antigens, as it has also been used to select for peptides that bind to two other small molecules, doxycycline and ecdysone, as well as cell surface determinants (K.M.M., R. Smith, and G.P.N., unpublished data).

We were able to test the structural requirement of the scaffold for high-affinity interaction with Texas red. The SKVILFE constraint is very important for high-affinity interaction with the dye in both TR501 and TR512. Replacing the SKVILFE constraint with a widely used cysteine constraint resulted in a greater than 10-fold loss in binding. There are two interesting explanations for this effect. First, it is possible that the cysteine constraint does not achieve the same orientation of the insert or that it forces the peptide insert into a shape that is not as suitable for binding to Texas red. Or, the SKVILFE region of the peptides might play a more active role and might interact with Texas red and thus help lead to high-affinity interactions, as noted above. This effect might be broadly useful in generating higher-affinity peptides via peptide library screening, as the vast majority of target proteins contain a combination of hydrophobic and hydrophilic character. This strategy has been applied to interactions between peptides and SH2 domains, where it had previously been difficult to achieve adequate affinity to study the interaction [25]. Thus, by presenting the aptamers in a context more like native proteins, we can achieve interactions that are more on the scale of typical protein-protein interactions, as opposed to the normally weaker peptide-protein interaction. In this case, we appear to have achieved this tighter interaction without sacrificing specificity.

The SKVILFE dimerized peptide library could be an ideal peptide library system, by virtue of its small size (a minimal SKVILFE scaffold is only 14 amino acids plus the randomized insert), its ability to form stable structures across a range of physiologic settings, and its propensity to permit high-affinity interactions between inserted peptides and targets. Other engineered protein scaffolds have been used in phage display, but none combine these three crucial features. Single-chain antibodies (scFvs) are used widely, but in this case, the scaffold is typically approximately 30 kDa (compared to 3 kDa for our SKVILFE domain), and scFvs typically fold poorly in the reducing environment of the cytosol [19].

We do not observe binding between these Texas red-selected peptides and fluorophores such as Fluorescein, which contains a xanthene core but lacks the exocyclic nitrogens. Thus, peptides TR501 and TR512 retain a great deal of selectivity, as they can distinguish between highly related molecules. Interestingly, TR501 and TR512 have different tolerance for variations in Texas red. TR512, the 9 amino acid peptide, binds to a broad range of Texas red derivatives and tolerates any changes in Texas red that conserve the xanthene core and exocyclic nitrogens. In contrast, TR501, which is 4 amino acids longer, is more sensitive to changes in Texas red outside of the xanthene core. Because TR501 has a longer insert, steric hinderance might impede binding of the RxxWEP region of the peptide to the core of Texas red when there is a large substituent present in the R region of Texas red.

### In Vivo Protein Labeling with X-Rhod-FF

Intracellular labeling of proteins with X-rhod-5F accomplishes several important goals. First, it demonstrates the potential to use the SKVILFE library to target a wide variety of fluorescent dyes, enabling the creation of a toolset that encompasses the diversity and sensitivity of organic fluorescent dyes. Second, it demonstrates a potential for Texas red-based dyes as a label in multicolor fluorescence applications in living cells. In this work, standard optical filters were used to analyze GFP fluorescence and Texas red fluorescence coherently. Red fluorescence is an ideal complement to the standard green fluorescence reagents, and red fluorescent dyes have excellent spectral separation from the autofluorescence of biological samples, which is predominantly in lower wavelengths [26]. Red Fluorescent Proteins (RFPs) have been cloned from various species, but these proteins tetramerize, typically causing precipitation of the RFP-target protein fusion, and alter the biology they are intended to detect [27]. While a monomeric RFP was recently created via mutagenesis, the protein suffered a strong loss of fluorescence intensity to remove the multimerization [28]. Texas red, on the other hand, is a bright dye with high quantum yield. An issue for Texas red in whole-cell imaging is its propensity to localize to mitochondrial organelles. Thus, localization assays with the current system must focus on protein sensing that does not overlap with such organelles or that such background can be visually masked or subtracted. It is likely that to fully appreciate the power of this approach will require the selection of peptides against dyes that do not have a propensity to localize within cells to any great degree.

Addressing this latter point, the interaction of TR512 with X-rhod-5F was tested in vivo because many, if not all, physiologic calcium signaling events are spatially restricted, and genetically targeted calcium sensors with such a large dynamic range could prove useful as probes in applications in cell biology and drug discovery [29]. Since X-rhod-5F has a Kd for $Ca^{2+}$ of 1.6 $\mu$M, the dye should be largely nonfluorescent in the cytosol (resting $Ca^{2+}$ of 100 nM) and is expected to undergo a large increase in fluorescence signal upon cytoplasmic calcium flux. Such increases are observed in electrical stimulation of neurons [30] and muscle [31] and upon mobilization of intracellular calcium stores [32]. Targeted calcium dyes would permit high-resolution detection and quantitation of these calcium signals, at a resolution far better than that achieved by calcium measurement by a whole-cell calcium indicator. For example, whole-cell calcium indicators have failed to measure the high calcium transients that are hypothesized to exist near the openings of active calcium channels due to saturation of the indicator and the transient nature of the calcium flux [33]. In efforts to address these needs, several genetically encoded calcium sensors have been created, using either fluorescence resonance energy transfer (FRET) between fluorescent proteins or by introducing a calcium binding element into GFP. The "Cameleon" proteins consist of two fluorescent proteins that flank a $Ca^{2+}$ binding site from Calmodulin and an M13 peptide that binds $Ca^{2+}$-Calmodulin. This causes a structural change upon $Ca^{2+}$ binding, causing an increase in FRET [9]. This system has only a 1.5-fold dynamic range, making quantification of calcium levels difficult, and it is 20 times larger than our Texas red targeting peptides (70 kDa to 3.5 kDa). Several groups have inserted a calcium

binding domain from Calmodulin into GFP or Yellow Fluorescent Protein (YFP), resulting in indicators with 2- to 4-fold calcium sensitivity, but these proteins are more sensitive to pH than to calcium [34, 35]. X-rhod-based dyes are available with a wide range of binding affinities for calcium, and thus a wide range of concentrations of calcium that they can measure. This wide range is crucial, because cellular calcium levels can vary vastly, from approximately 100 nM in the cytoplasm to 100–400 μM in the endoplasmic reticulum. Future work might explore these other X-rhod dyes or other dye systems and test their ability to measure a wide range of calcium signals in cells in more detail.

## Significance

**In summary, a peptide aptamer that binds to Texas red and the calcium sensor X-rhod was selected using a novel, constrained phage display library. The peptide does not interfere with the salient fluorescence properties of target dyes, and it permits protein labeling in vitro and in vivo with organic small molecule dyes. Future work might include applications where TR512 is used to target X-rhod dyes to proteins or subcellular areas wherein local calcium levels fluctuate for physiologically interesting reasons. In addition, this peptide library system could be used to select for peptides that bind to other fluorescent dyes, including molecules with different colors or molecules that sense other important biophysical properties like hydrophobicity or pH.**

### Experimental Procedures

#### Peptide Library Creation

9 and 13 amino acid peptide library random regions were constructed in two steps. First, oligonucleotides were generated which contained sequences coding for a repeat of a homodimerization sequence SKVILFE on either side of FseI and AscI sites. This cassette was flanked by KpnI and EagI sites, which were used to clone the SKVILFE cassette into the phage display vector PhD121 (New England Biolabs). Next, duplex DNA was generated by annealing and extension of two oligonucleotides to yield a duplexes with the sequence 5′-CCTCTACACATCCATGGGCCGGCCGGA(NNK)$_{9/13}$GGCGCGCCTTCCACC-3′, where the underlined sequences represent FseI and AscI sites, N represents an equal mixture of all four nucleotides, and K represents an equal mixture of G and T. Restricting the third nucleotide of each codon in the library insert reduces the likelihood of a stop condon (from 3/64 = .0468 to 1/32 = .03125) while still allowing all 20 amino acids to be present in the library. This duplex DNA was cloned into the phage vector using the FseI and AscI sites and transformed into bacteria, generating $3.1 \times 10^8$ unique inserts for the 9-mer library and $1.2 \times 10^8$ unique inserts for the 13-mer library. The resulting phages are expected to express five copies of the peptide at the N terminus of the g3p.

#### Selection and Conjugation to Solid Support

To select for phage that bind to fluorescent dyes, the Texas red and rhodamine red (Molecular Probes) were chemically coupled to a Diaminodipropylamine (DADPA) column as described previously [16]. The 9-mer and 13-mer libraries were mixed, and $8.7 \times 10^{11}$ phage were used as the input for the first round of panning. Subsequent rounds used $4.5 \times 10^{11}$ phage. In each round of selection, phage were incubated with 150 μl of dye-conjugated column in 10 ml of TBS with .1% Tween and 2 mg/ml BSA for 4 hr, then washed with 100 bed volumes of TBS/Tween, and bound phage eluted with 0.2 M Glycine (pH 2.2). These phages were amplified and used as input in the next round of screening. Five rounds of screening were performed, at which point clones were selected for characterization.

#### Phage-Dye Kd Determination

Binding constants for the interaction between the selected phage clones and Texas red were determined by incubating Texas red-DADPA beads with phage clones at dilutions ranging from $3 \times 10^7$ to $9 \times 10^9$ phage/ml for 1 hr at 37°C. The fraction of bound versus unbound phage was measured at least six separate times for each phage concentration. Scatchard analysis was performed to determine the binding constant via standard calculations.

#### In Vitro Binding of His-Tagged Peptides and Texas Red or X-Rhod Dyes

Peptides were synthesized to >95% purity (American Peptide Company). Six histidine residues were added to the C terminus to permit detection and immobilization of the peptides, resulting in 48-mer (TR501) and 44-mer (TR512, control peptide) peptides. The linear peptide (TR401) has the sequence KHVQYWTQMFYSGGGSAETVG GGHHHHHH; the control peptide has the sequence GGGSKVILFEG PAGPSGSAGSGASGAPGSKVILFEGGPGHHHHHH; and peptides TR501 and TR512 are identical to the control peptide, except that the sequences RTIWEPKEASNHT and RWTWEPISE replace the internal sequence SGSAGSGAS. For in vitro binding assays, his-tagged peptides were bound to Talon cobalt resin (Clontech) by incubation of 1 μM peptide and 10 μl of Talon resin for 30 min at room temperature. After three washes, Texas red, or a Texas red derivative, was added to 500 nM and incubated for 30 min at room temperature. Free dye was removed during three washes, and Texas red bound to peptide-loaded beads was detected with an Axiophot II fluorescent microscope equipped with a Texas red filter set (BP: 546/12, FT: 580, LP: 590). All images were collected using identical settings within the linear range of detection, and fluorescence intensity per bead was quantified using NIH Image or OpenLab software. The linearity of the assay was established by quantitation of the fluorescence intensity of known concentrations of Texas red in solution. Beads with smaller and more uniform diameter (Bangs Laboratories) were used in an identical protocol and analyzed for Texas red fluorescence with a Vantage FACS machine.

#### Peptide Epitope Mapping and GST-Peptide Creation

C-terminal fusions of the peptides to GST were generated, using recombination-based cloning and the pDEST15 vector (Invitrogen). The GST fusions were designed with the general sequence "GST-GGGSKVILFEGPAG-insert-GAPGSKVILFEGGPG-stop." Derivatives containing a disulfide bridge instead of the SKVILFE constraint had the general sequence "GST-GGGCGPAG-insert-GAPGCggpg-stop." Linear derivatives possessed the general sequence "GST-GGGGPAG-insert-GAPGGGPG-stop." GST fusions with the control peptide contain the insert "SGSAGSGAS," fusions with TR501 contain the insert "RTIWEPKEASNHT," and fusions with TR512 contain the insert "RWTWEPISE." GST fusion peptides were expressed in BL21 bacteria and purified to 90%–95% purity using Glutathione Sepharose 4B. Standard ELISAs were performed, where a Texas red-BSA conjugate was adsorbed to 96-well plates. Various GST-peptides were permitted to bind, washed extensively, and detected with an anti-GST HRP antibody (Amersham Pharmacia) and ABTS substrate measured by its absorbance at 405 nm.

#### Intracellular Staining with X-Rhod-FF

Retroviral constructs were made containing a three-part fusion with a 10 amino acid plasma membrane targeting sequence from Lyn, GFP, and either TR512 or control peptide. NIH3T3s were infected and stable cell lines were generated by selection with Blasticidin driven by an internal ribosome entry site (IRES). Cells were plated on coverslips 1 day prior to imaging experiments. To stain cells, 1 μM X-rhod-5F-am was incubated with cells for 30 min at 37 degrees. Cells were then washed and incubated at 37 degrees for 1 hr prior to imaging. 10 μM ionomycin was added 3–5 min before imaging. Cells were imaged using a Zeiss LM510 confocal microscope, and all images were acquired with identical microscope settings. 1 μm thick confocal sections were scanned through the midsection of cells to ensure appropriate distinction of plasma membrane and

cytosolic staining. Quantitation of relative fluorescence intensities was performed using NIH Image.

**Acknowledgments**

The authors would like to acknowledge funding from the National Institutes of Health, a Stanford Graduate Fellowship to K.M.M., and the Robert Wood Johnson Foundation. The authors would also like to thank Bruce Cohen for critical review of the manuscript.

Received: November 1, 2003
Revised: December 6, 2003
Accepted: December 12, 2003
Published: March 19, 2004

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