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Lanthanide-Binding Tags as Luminescent Probes for Studying Protein Interactions

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Abstract: Herein, we report a method for studying protein−peptide interactions which exploits the luminescence properties of Tb(III). Lanthanide-binding tags (LBTs) are short peptide sequences comprising 15−20 naturally occurring amino acids that bind Tb(III) with high affinity. These genetically encodable luminescent tags are smaller in size than the *Aequorea victoria* fluorescent proteins (AFPs) and benefit from the long-lived luminescence lifetime of lanthanides. In this study, luminescence resonance energy transfer (LRET) was used to monitor the interaction between SH2 domains and different phosphopeptides. For the study, the SH2 domains of Src and Crk kinase were each coexpressed with an LBT, and phosphorylated and nonphosphorylated peptides were chemically synthesized with organic fluorophores. The LRET between the protein-bound Tb(III) and the peptide-based organic fluorophore was shown to be specific for the recognition of the SH2 domain and the peptide binding partner. This method can detect differences in binding affinity and can be used to measure the dissociation constant for the protein−peptide interaction. In addition, decay experiments can be used to calculate the distance between a site in the bound peptide and the protein using Förster theory. In all of these experiments, the millisecond luminescence lifetime of Tb(III) can be exploited using time-resolved detection to eliminate background fluorescence from organic fluorophores.

Introduction

It is widely accepted that protein−protein interactions are central to the orchestration of biological complexity.[1] Complementary chemical and biological approaches to detect and study protein−protein association will provide a deeper understanding of the nature, regulation, and function of these interactions, which is central to human health.[2,3] Fluorescence spectroscopy has become a powerful tool for monitoring biological events and elucidating the structure and function of biomolecules.[4] The sensitivity and time-resolved nature of fluorescence, in addition to the ready availability of necessary equipment, has made fluorescence spectroscopy a popular choice for studying biological processes. Recently, lanthanides have found wide success as luminescent probes.[5,6] Due to their long (millisecond) luminescence lifetimes, sharp emission spectra with large Stokes shifts (>200 nm), and unpolarized luminescence, lanthanide ions demonstrate features not common in the typical organic fluorophores.

Recent advances in using lanthanides as biological probes have included detection of conformational changes in Cytoplasmic Domain of Human Anion Exchanger 1,[7] clarification of disputes regarding K^+ channel movement,[8] development of a saccharide biosensor,[9] and detection of flexibility in Tropomyosin.[10] In all these examples, the lanthanide ion is incorporated into the protein either by using an intrinsic metal ion-binding loop or by chemically modifying a nucleophilic amino acid in the protein with an electrophilic derivative of a chelate. While excellent results were obtained from these experiments, they are limited to studies of proteins with intrinsic metal-ion-binding sites or require chemical modification procedures that can be unreliable and often nonspecific. A general method for tagging proteins with lanthanide ions will facilitate their use as luminescent probes and further applications of lanthanide-ion-based technologies.

Pioneering work by Szabo and co-workers demonstrated that calcium-binding loops with an appropriately placed tryptophan residue serve as luminescent Tb(III) probes when tagged onto a protein of interest.[11,12] The indole ring of the tryptophan serves as a sensitizer, to overcome the inherently low absorbance of the Tb(III). The ease with which these short sequences can be incorporated into proteins by standard molecular biology

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techniques would make them ideal lanthanide-binding tags; however, they suffer from poor Tb(III) affinity. Recently, we reported a combinatorial screen to discover peptides with a high affinity for Tb(III) using a luminescence-based selection strategy.[13−15] This strategy led to the identification of a lanthanide-binding tag (LBT) of only 20 amino acid residues with a low nanomolar dissociation constant for Tb(III). The small size of the LBTs, compared to the significantly larger *Aequorea victoria* fluorescent proteins (AFPs), and the long-lived luminescence lifetime make the LBTs an appealing complement to existing encoded fluorescent protein tags for in vitro studies.[16]

The emergence of luminescent tags for biomolecules has fueled the effort to expand the power of spectroscopic methods, such as resonance energy transfer (RET).[17,18] The most common of these experiments is fluorescence resonance energy transfer (FRET), in which an excited donor fluorophore transfers energy to an acceptor fluorophore. Since lanthanide emission is technically not fluorescence (as it does not result from a singlet-to-singlet transition), the related process is called luminescence resonance energy transfer (LRET). RET can be monitored by studying the emission of the acceptor and is a function of the proximity of the two fluorophores. For this reason, LRET can be used to measure inter- and intramolecular distances as well as to detect two interacting moieties. In LRET, the millisecond luminescence lifetime of the lanthanide ion can be exploited by collecting the emission after a short time delay (50 μs after excitation).[19,20] This time delay eliminates any background luminescence from direct excitation of the acceptor, which is a common complication in FRET measurements.

In this study, the versatility of the LBT is demonstrated by using LRET to monitor the interactions between Src homology 2 (SH2) domains and phosphopeptides. SH2 domains are small protein domains of approximately 100 amino acids that are characterized by specific binding to peptides that include phosphotyrosine residues. This phosphorylation-specific interaction is involved in numerous signal transduction pathways.[21−24] The association of these small protein domains is determined primarily by the phosphotyrosine residue, but the adjacent residues are essential for selectivity, which has been demonstrated by screens of peptide libraries[25] and visualized in the bound and unbound forms of the SH2 domain crystal structure.[26] Additional studies have been performed to show that specific binding to the SH2 domain can be achieved with small peptide sequences.[25,27] Herein we describe studies on this important biological association which demonstrate the applicability of LBTs as luminescent probes for monitoring protein−peptide interactions as well as for measuring the distance between specific sites on the SH2 domain and an interacting phosphopeptide (Figure 1a).

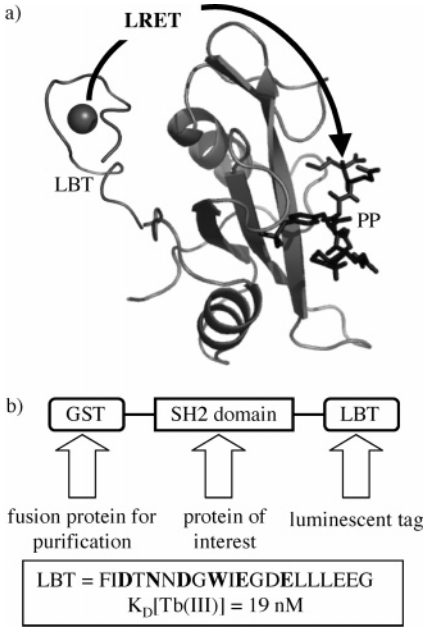

Figure 1. (a) Graphic of Src-LBT with a bound phosphopeptide (generated from the crystal structure using PyMOL, PDB accession code 1SPS). LRET is used to detect the interaction and measure the distance between the Tb-(III) and an organic fluorophore on the phosphopeptide (PP). (b) Protein construct with N-terminal GST and C-terminal LBT.

Results and Discussion

The LBT was encoded at the DNA level C-terminal to the SH2 gene via polymerase chain reaction using a primer encoding the LBT gene. The DNA was inserted into a pGEX plasmid (Amersham Biosciences) to generate a glutathione S-transferase (GST) fusion protein, which affords high expression levels and facilitates purification (see Supporting Information for details). It has previously been demonstrated that an N-terminal GST fusion protein does not interfere with SH2-binding interactions.[28] The protein was overexpressed in *Escherichia coli* to provide the SH2 domain with an N-terminal GST and a C-terminal LBT (see Figure 1b). This procedure was followed for the SH2 domains of both Src and Crk kinases to test for phosphopeptide-binding specificity. The LBT chosen for this study comprises 20 amino acid residues and is the tightest non-disulfide-containing LBT for Tb(III) reported to date,[15] with a 19 nM binding constant in the free peptide form.[13] The peptide sequence, FI**D**T**N**N**D**G**W**I**E**GD**E**LLLEEG, includes six coordinating residues (shown in bold) and the tryptophan residue, which is crucial for Tb(III) sensitization and also provides a backbone carbonyl oxygen as a ligand.[29] Previous spectroscopic and crystallographic studies in the group have demonstrated that the inner coordination sphere of Tb(III) is free from bound water

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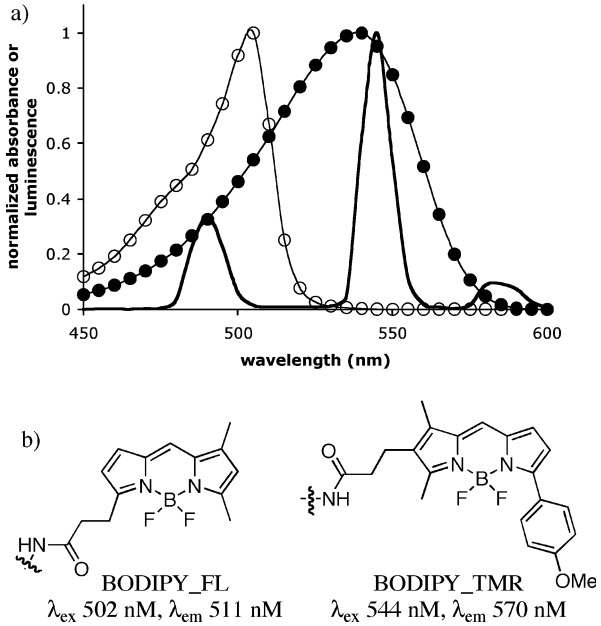

Figure 2. (a) Spectral overlap of LBT emission (—) and the absorbance of BODIPY_FL (○) and BODIPY_TMR (●). (b) Chemical structures of BODIPY_FL and BODIPY_TMR.

Table 1. Peptide Sequences That Bind Src and Crk SH2 Domains[a]

peptide	peptide sequence
Src-PP	Glu-Pro-Gln-**pTyr-Glu-Glu-Ile**-Pro-Ile-Tyr-Leu-$CONH_2$
Src-CP	Glu-Pro-Gln-Tyr-Glu-Glu-Ile-Pro-Ile-Tyr-Leu-$CONH_2$
Crk-PP	Gln-**pTyr-Asp-His-Pro**-Asn-Ile-$CONH_2$
Crk-CP	Gln-Tyr-Asp-His-Pro-Asn-Ile-$CONH_2$

[a] Residues implicated in binding are shown in bold.

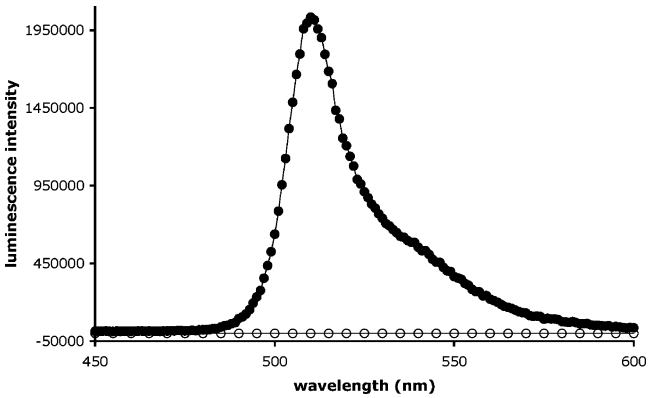

Figure 3. Emission spectrum of BODIPY_FL-Src-PP collected via excitation at 280 nm; no delay (●) and 50 μs delay after flash (○).

molecules,[29] which is especially important for luminescence experiments, since the O−H vibration can quench the Tb(III) luminescence.[30]

Before commencing with LRET experiments, it was important to confirm that appending the LBT to the protein would not negatively impact its affinity for Tb(III). Both GST-Src-LBT and GST-Crk-LBT were directly titrated with Tb(III), and the sensitized luminescence intensity was monitored at 544 nm (see Materials and Methods section for details). Both constructs showed excellent Tb(III) affinity, with a 25 nM binding constant for GST-Src-LBT and a 5 nM binding constant for GST-Crk-LBT. Thus, at low micromolar concentrations, only 1 equiv of Tb(III) is necessary to ensure complete lanthanide loading. This removes the need for excess lanthanide that may perturb the biological system through nonspecific binding.

Next, an appropriate acceptor was chosen to label the phosphopeptides. While many organic fluorophores have spectral overlap with one of the three Tb(III) emission bands at 490, 545, and 590 nm (see Figure 2), the 4,4-difluoro-4-bora-3a,4a-diaza-*s*-indacenes (BODIPYs) have emerged as a powerful fluorophore family. BODIPYs are commercially available with amine- or thiol-reactive functional groups for labeling both peptides and proteins. They have high photostability at pH > 2, large extinction coefficients (ϵ_{max} up to 100 000 M^{-1} cm^{-1}), and high quantum yields. For this study, two BODIPY fluorophores were chosen, BODIPY_FL and BODIPY_TMR, which overlap with the Tb(III) emission at 490 and 544 nm, respectively (see Figure 2). These fluorophores are named on the basis of their having excitation and emission wavelengths similar to those of fluorescein and tetramethylrhodamine, respectively.

As stated earlier, SH2 domains are characterized by binding to specific phosphotyrosine-containing sequences; therefore, phosphopeptides (**PP**) known to bind to Src and Crk SH2 domains were selected for our studies (**Src-PP** and **Crk-PP**, see Table 1).[25] All peptides were synthesized by solid-phase peptide synthesis (SPPS) followed by chemical labeling with the appropriate BODIPY derivative, using succinimidyl esters to react with the N-terminal amines (see Supporting Information for details). A 6-aminohexanoic acid linker was incorporated between the peptide and BODIPY to ensure that the fluorophore did not interfere with the peptide−protein interactions. Also, to test that the interaction was phosphorylation-dependent, control peptides (**CP**), in which the phosphotyrosine is substituted for tyrosine (**Src-CP** and **Crk-CP**), were also generated (see Table 1).

LRET Studies. All experiments were conducted in a Fluoromax-3 spectrometer equipped with a phosphorimeter to enable pulsed excitation followed by a delay after flash, thus eliminating background fluorescence from direct excitation of the acceptor fluorophore. The gated experiment is possible due to the millisecond luminescence lifetimes of lanthanides and highlights one of the benefits of LRET over typical fluorescence experiments such as FRET. To demonstrate the utility of this approach, a solution of BODIPY_FL-Src-PP was excited at 280 nm (the excitation wavelength for Tb(III) sensitization with tryptophan) with and without the 50 μs delay after flash. As shown in Figure 3, there is a significant amount of direct excitation of BODIPY_FL, which is completely eliminated by using the 50 μs gate. Thus, in time-resolved experiments, any signal detected at 510 nm is entirely due to resonance energy transfer from the LBT to the BODIPY_FL, and not from direct excitation. It should also be noted that, while excitation at 280 nm is not optimal for biological samples, we did not observe any deleterious effects in these in vitro experiments. Additionally, the luminescence experiments are conducted with pulsed excitation and not continual irradiation, which minimizes exposure.

Binding studies began by incubating the SH2 domains with phosphopeptides and collecting the luminescence spectra (see

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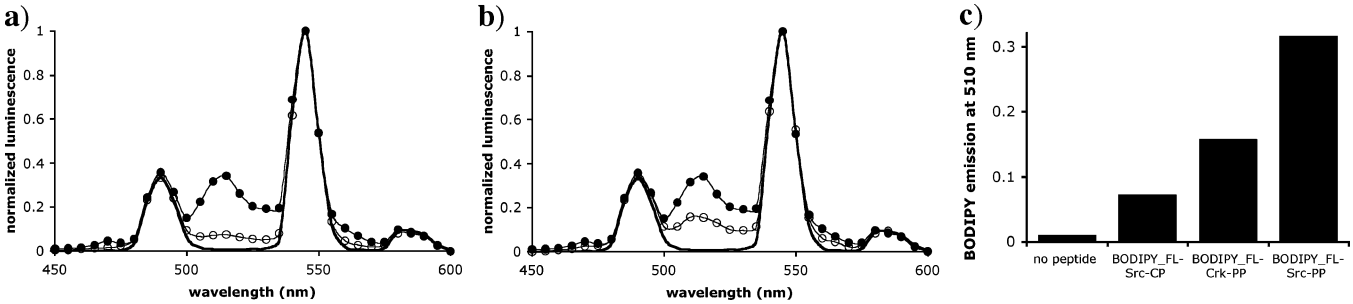

Figure 4. LRET detection as a function of binding to Src-SH2 domain. Samples were excited at 280 nm, and the gated emission was recorded from 450 to 600 nm. (a) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III) incubated with no peptide [—], 0.5 μM BODIPY_FL-Src-PP [●], or 0.5 μM BODIPY_FL-Src-CP [○]. (b) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III) incubated with no peptide [—], 0.5 μM BODIPY_FL-Src-PP [●], or 0.5 μM BODIPY_FL-Crk-PP [○]. (c) Intensity of the luminescence signal at 510 nm corresponding to energy transfer to BODIPY_FL.

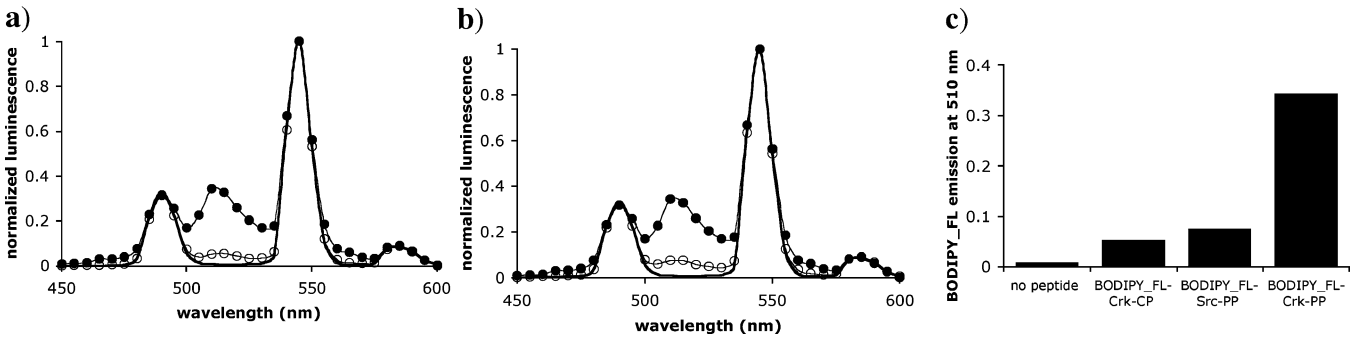

Figure 5. LRET detection as a function of binding to Crk-SH2 domains. Samples were excited at 280 nm, and the gated emission was recorded from 450 to 600 nm. (a) 0.5 μM GST-Crk-LBT, 0.5 μM Tb(III) incubated with no peptide [—], 0.5 μM BODIPY_FL-Crk-PP [●], or 0.5 μM BODIPY_FL-Crk-CP [○]. (b) 0.5 μM GST-Crk-LBT, 0.5 μM Tb(III) incubated with no peptide [—], 0.5 μM BODIPY_FL-Crk-PP [●], or 0.5 μM BODIPY_FL-Src-PP [○]. (c) Intensity of the luminescent signal at 510 nm corresponding to energy transfer to BODIPY_FL.

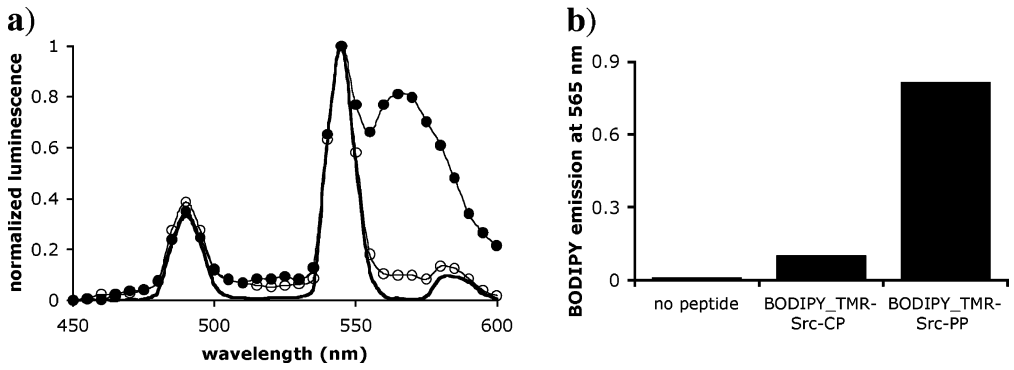

Figure 6. LRET detection as a function of binding to Src-SH2 domains. Samples were excited at 280 nm, and the gated emission was recorded from 450 to 600 nm. (a) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III) incubated with no peptide [—], 0.5 μM BODIPY_TMR-Src-PP [●], or 0.5 μM BODIPY_TMR-Src-CP [○]. (c) Intensity of the luminescence signal at 565 nm corresponding to energy transfer to BODIPY_TMR.

Materials and Methods section for details). A solution containing 0.5 μM GST-Src-LBT, Tb(III), and BODIPY_FL-**Src-PP** exhibited a large LRET peak at 510 nm (see Figure 4a [●]). This result is in contrast to the weak signal exhibited by the non-phosphorylated peptide BODIPY_FL-**Src-CP** (see Figure 4a [○]). In addition, SH2 domain specificity could be detected with different phosphotyrosine peptides. As shown in Figure 4b, a more intense LRET signal is detected for the Src-binding peptide [●] over the Crk-binding peptide [○]. This difference in LRET signal is illustrated more clearly in Figure 4c, which shows the largest LRET signal for **Src-PP**. It is also interesting to note that, as predicted, the most important contributor to recognition is the phosphotyrosine residue, demonstrated by the larger LRET signal for **Crk-PP** over **Src-CP** (Figure 4c).

GST-Crk-LBT was tested in a fashion identical to that used for the Src construct, and the results are shown in Figure 5. These experiments demonstrate that the method can detect selective interactions between different binding partners. The specificity of Crk for **Crk-PP** was even greater than the specificity of Src for **Src-PP** (Figure 5c), which is consistent with previous studies that show fewer binding sequences for Crk in comparison to Src.[25]

We also investigated an alternative acceptor that overlaps with a different Tb(III) emission band. For these studies, **Src-PP** and **Src-CP** were labeled with BODIPY_TMR. Performing the LRET binding assay as discussed previously provided encouraging results with a very large LRET signal at 565 nm (see Figure 6). The intensity of this LRET is larger than that of the LRET with BODIPY_FL due to the greater spectral overlap of BODIPY_TMR and the Tb(III) emission at 545 nm (see Figure 2).

Binding Constant and Competition Experiments. To ensure that the luminescent tags were neither interfering with

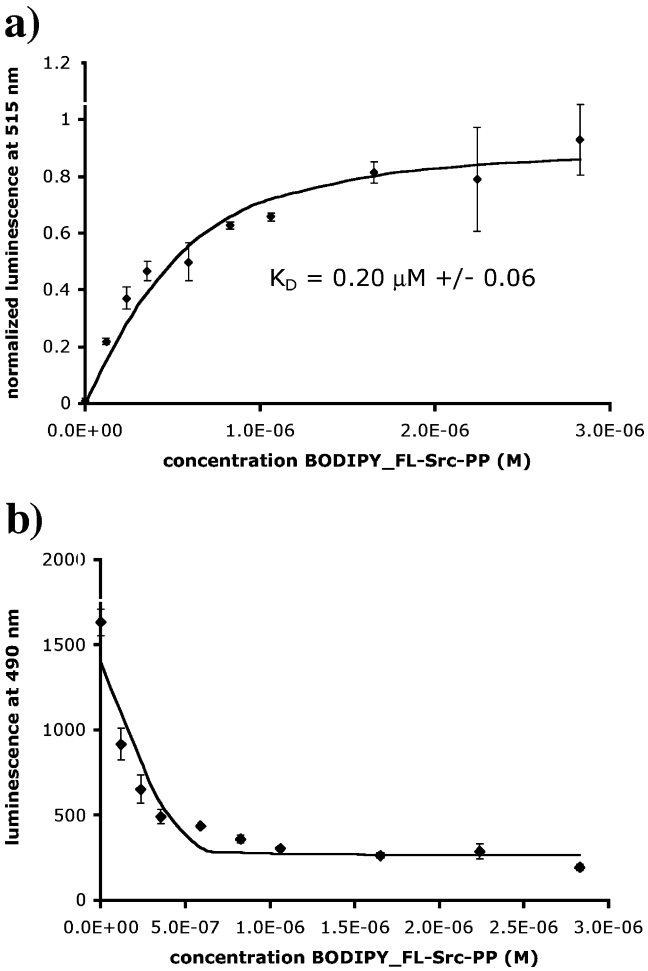

Figure 7. Titration of 0.5 μM GST-Src-LBT and Tb(III) with BODIPY_FL-Src-PP to obtain the binding constant for the peptide−protein interaction. (a) Normalized luminescence measured at 515 nm. (b) Un-normalized luminescence at 490 nm.

nor altering the binding interaction of the SH2 domain with the phosphopeptide, the binding constant for this interaction was determined. This was accomplished by monitoring the LRET signal of a solution containing 0.5 μM GST-Src-LBT and Tb(III) with increasing concentrations of BODIPY_FL-Src-PP. A plot of the LRET signal at 510 nm versus concentration of BODIPY_FL-**Src-PP** was fit using SPECFIT[31] to determine the binding constant (see Figure 7a). This method provided a binding constant of 0.20 ± 0.09 μM, which is consistent with the previously reported binding constants of 0.77 and 0.40 μM for GST fusion Src SH2 domains using isothermal calorimetry and surface plasmon resonance, respectively.[28] However, it should be noted that it is difficult to accurately measure binding constants when the concentration is approaching the K_D. LRET can also be detected by a decrease in the luminescence of the donor, which is seen in the un-normalized luminescence data at 490 nm (see Figure 7b).

To further ensure that the interaction between the LBT-labeled protein and the BODIPY-labeled peptide is specific and reversible, competitive binding experiments were performed.

(31) Binstead, R.; Jung, B.; Zuberbuhler, A. *SPECFIT/32 for Windows* (version 3.0.30), S.S.A., Marlborough, MA. SPECFIT is owned solely by its authors. SPECFIT provides global analysis of equilibrium and kinetic systems using singular value decomposition and nonlinear regression modeling by the Levenberg−Marquardt method.

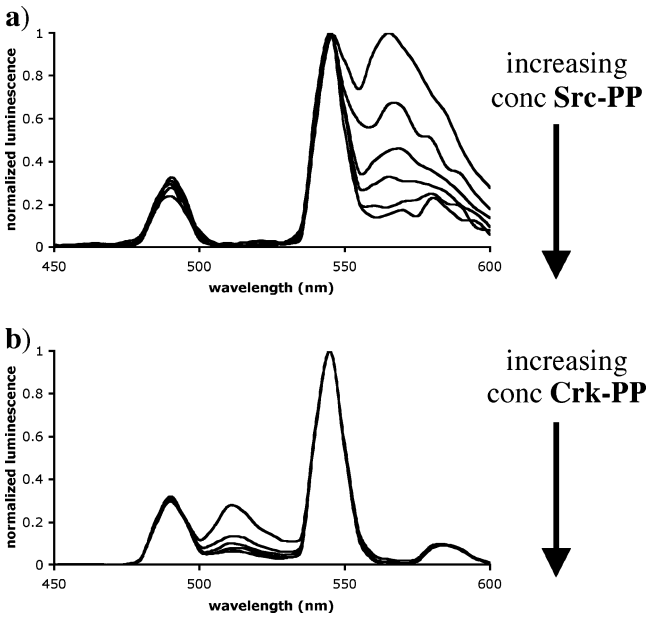

Figure 8. Competition experiments conducted by excitation at 280 nm followed by time-resolved emission collection (50 μs) from 450 to 600 nm. (a) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III), and 0.5 μM BODIPY_TMR-Src-PP with increasing concentration of Src-PP. (b) 0.5 μM GST-Crk-LBT, 0.5 μM Tb(III), and 0.5 μM BODIPY_FL-Crk-PP with increasing concentration of Crk-PP.

Addition of unlabeled phosphopeptide to the protein with bound BODIPY-labeled phosphopeptide should result in a continual decrease in LRET signal. This experiment was performed with both GST-Src-LBT and GST-Crk-LBT and, as anticipated, with increasing concentrations of unlabeled **Src-PP** and **Crk-PP**, the LRET signal decreased (see Figure 8). Control experiments involving addition of only buffer demonstrated that the LRET decrease was due to displacement of the BODIPY-labeled peptide and was not an artifact of photobleaching. Both the titration and competition experiments demonstrate that the LRET signal is specific and reversible and is solely due to the recognition of the phosphotyrosine peptide by the SH2 domain.

Decay Experiments and Distance Measurements. The power of LRET is in its ability not only to detect and quantify binding interactions but also to provide information on the distance between the binding partners. The millisecond luminescence lifetime of Tb(III) allows decay measurements to be conducted using simple, commercially available equipment. The luminescence lifetime (τ) that is derived from the decay data is used to calculate the distance between the donor and acceptor. These measurements were conducted with excitation of the sensitizing tryptophan at 280 nm, followed by collection of the emission intensity at either 490 or 545 nm, with an initial delay of 50 μs (see Materials and Methods section for details). In the presence of the acceptor, the RET to the acceptor will affect the Tb(III) luminescence lifetime. As shown in Figure 9, the decay rate is faster for both GST-Src-LBT and GST-Crk-LBT in the presence of the BODIPY-labeled peptides (● versus ○). Using SIGMAPLOT, the curves were fit to a monoexponential [$I(t) = I(0)\ e^{(-t/\tau)}$] in the absence of the acceptor, and to a biexponential [$I(t) = I(0)_1\ e^{(-t/\tau 1)} + I(0)_2\ e^{(-t/\tau 2)}$] in the presence of the acceptor, where $I(t)$ is the luminescence intensity at time t after the excitation pulse, $I(0)$ is the initial intensity at $t = 0$,

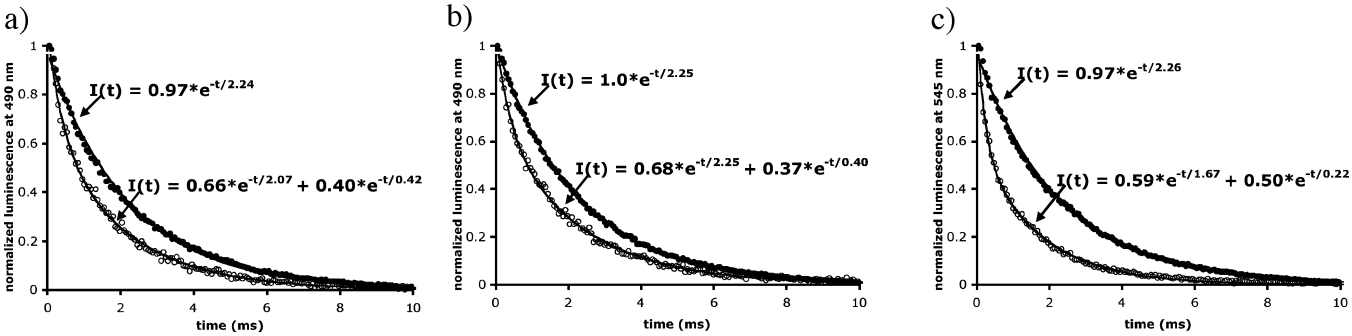

Figure 9. Solutions were excited at 280 nm with an initial gate of 50 μs and the luminescence decay was recorded in 60 μs increments. (a) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III) (no acceptor [●]; 0.5 μM BODIPY_FL-Src-PP [○]). (b) 0.5 μM GST-Crk-LBT, 0.5 μM Tb(III) (no acceptor [●]; 0.5 μM BODIPY_FL-Crk-PP [○]). (c) 0.5 μM GST-Src-LBT, 0.5 μM Tb(III) (no acceptor [●]; 0.5 μM BODIPY_TMR-Src-PP [○]).

Table 2. LRET Data for Calculating Distances

protein	peptide	J (M^{-1} cm^{-1} nm^4)	R_0 (Å)	energy transfer	R (Å)
GST-Src-LBT	BODIPY_FL-Src-PP	5.3×10^{14}	39.6	0.81 ± 0.13	31.1 ± 5.0
GST-Src-LBT	BODIPY_TMR-Src-PP	2.4×10^{15}	50.9	0.90 ± 0.02	35.3 ± 1.3
GST-Crk-LBT	BODIPY_FL-Crk-PP	6.2×10^{14}	40.6	0.82 ± 0.07	31.5 ± 2.5

and τ is the lifetime.[32] All curves were fit to $r^2 = 0.99$ and showed very little residual structure.

LRET follows the same principles as FRET; thus, Förster theory can be applied for calculating distances between the acceptor/donor pair.[33] Förster theory relates the resonance energy transfer to the distance according to eq 1, where E is the energy transferred, R_0 is the Förster distance, and R is the distance between the donor and acceptor. The percentage of energy transferred (E) can be derived from the lifetime measurements by the relationship shown in eq 2, where τ_D is the lifetime of the donor alone and τ_{DA} is the lifetime of the donor in the presence of the acceptor.

$$R = R_0[(1/E) - 1]^{1/6} \quad (1)$$

$$E = \frac{1 - \tau_{DA}}{\tau_D} \quad (2)$$

From the donor-only decay plot of GST-Src-LBT, $\tau_D = 2.24$ ms (see Figure 9a). The biexponential fit of the luminescence decay of GST-Src-LBT in the presence of BODIPY_FL-**Src-PP** provided two lifetimes. The 2.07 ms component corresponds to the donor only and is not influenced by the acceptor, whereas the 420 μs component is due to energy transfer.[10] Hence, in eq 2, $\tau_{DA} = 0.42$ ms and yields an energy transfer of 81%. The same procedure was followed to calculate the energy transfer for the other constructs, the data of which are shown in Table 2.

The Förster distance (R_0) must be derived for each acceptor/donor pair and is the distance at which there is 50% energy transfer ($E = 0.5$). R_0 is calculated by the relationship shown in eq 3.

$$R_0 = 0.211(\kappa^2 \eta^{-4} Q_D J) \quad (3)$$

$$J = \frac{\sum[F_D(\lambda)\epsilon(\lambda)\lambda^4\Delta\lambda]}{\sum[F_D(\lambda)\Delta\lambda]} \quad (4)$$

The orientation factor (κ^2) is most commonly taken as 2/3 for LRET measurements and assumes a randomized orientation for both donor and acceptor.[32] The quantum yield of the donor (Q_D) is most easily obtained from τ_D/τ_{Tb}, where τ_D is the lifetime of the donor and τ_{Tb} is the lifetime of free Tb(III) when directly excited with a laser (4.75 ms).[34] The refractive index (η) is taken as 1.4 for biological samples in H_2O. The spectral overlap (J) is a measure of the overlap of the emission spectra of the donor and absorption spectra of the acceptor and is calculated using eq 4 for each donor/acceptor. $F_D(\lambda)$ is the corrected fluorescence intensity of the donor, and $\epsilon(\lambda)$ is the extinction coefficient of the acceptor. As expected, the J value for the LBT and BODIPY_TMR is greater, due to the larger spectral overlap of this fluorophore pair (see Table 2).

Utilizing eq 1, the calculations reveal that the distance between the LBT and BODIPY is approximately 31−35 Å. An important internal control was measuring the same protein−peptide pair with a different acceptor to show that the calculations were consistent, since there is a negligible size difference between the two fluorophores. When GST-Src-LBT was used with both BODIPY_FL and BODIPY_TMR, less than 5 Å difference was calculated. This is consistent with reported studies using different fluorophore pairs, particularly considering the significant difference in J values between the two acceptors (see Table 2).[32]

Conclusions

In this study, the SH2 domains of Src and Crk kinase were genetically encoded with an LBT to study binding interactions using LRET. Phosphorylated and non-phosphorylated peptides were chemically labeled with BODIPY fluorophores, which serve as the LRET acceptor. The interaction of different phosphopeptides and SH2 domains was detected by LRET and shown to be specific for the binding interaction. This method

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can detect differences in binding affinity and showed a large luminescence signal for the cognate phosphopeptide. In contrast, a very low signal was obtained for the non-phosphopeptide or a phosphopeptide with a mismatched sequence. The LBT and BODIPY tags did not interfere with the protein−peptide association, and LRET was used to calculate the dissociation constant for the interaction.

The advantages of the millisecond luminescence lifetime of Tb(III) were highlighted by the collection of decay data on a standard fluorometer. This decay data was used to measure the distance between the protein and bound peptide. Förster theory provided a distance of 31−35 Å between the Tb(III) and BODIPY. As a control, two different BODIPY fluorophores were used to measure the distance. Good agreement was seen in the distance calculations, even with the large difference in spectral overlap between the two systems.

The developed LRET method is also amenable to screening protein−protein interactions, with the continual development of improved methods to chemically label proteins with fluorophores.[35,36] Continued development may also expand this in vitro screening method into cellular-based assays. As methods for shuttling lanthanides into cells are developed through either ion channels,[37,38] masked cargo,[39] or new technology, researchers will be able to take full advantage of this small, coexpressed tag. With the basic strategy presented, current work will seek to address alternative approaches to the short excitation wavelength currently necessary for Tb(III) sensitization. Continued work in this area may ultimately provide a complement to the widespread successful use of AFPs as in vivo probes.[40−43]

Materials and Methods

Terbium Titrations. Luminescence titrations were conducted on a Horiba Jobin Yvon Fluoromax-3 in 1 cm path length quartz cells. Sensitization of Tb(III) luminescence was carried out by excitating tryptophan at 280 nm and recording the luminescence at 544 nm. A 315 nm long-pass filter was used to eliminate interference from harmonic doubling. Excitation and emission slit widths of 5 nm were used with 1 s integration times. Spectra were corrected for emission intensity by using manufacturer-supplied correction factors. Spectra were recorded at pH = 7 in 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) buffer and 100 mM NaCl. Aliquots of Tb(III) ions were added, and the luminescence was recorded to obtain a titration curve. These data were fit using the program SPECFIT[31] to derive the Tb(III) binding constant. Data are the average of five runs.

LRET. Time-gated experiments were recorded on a Horiba Jobin Yvon Fluoromax-3 equipped with a Spex 1934D3 phosphorimeter. The sample was excited at 280 nm with a lamp pulse followed by a delay after flash of 50 μs. A luminescent scan was then recorded from 450 to 600 nm with 5 nm increments. The sample window for data collection was 10 ms, with time per flash of 40 ms and 100 flashes recorded per reading. LRET data were obtained in 10 mM 2-(*N*-morpholino)-ethanesulfonic acid (MES) buffer and 100 mM NaCl, pH = 6.9. Reported data are the average of three runs. Luminescence spectra were normalized for visualization of different data intensities and to provide a contrast enhancement of the LRET signal.

Decay Measurements. Lifetime measurements were recorded on a Horiba Jobin Yvon Fluoromax-3 equipped with a Spex 1934D3 phosphorimeter. The intensity at 490 or 544 nm was monitored at 60 μs increments for 12 ms after an initial delay of 50 μs, following a lamp pulse at 280 nm from a xenon flash lamp. Decay measurements were performed in 10 mM MES buffer and 100 mM NaCl, pH = 6.9. Reported data are the average of three runs.

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Supporting Information Available: Detailed experimental procedure on peptide synthesis, BODIPY-labeling, genetic encoding of LBT, protein expression and purification, and complete ref 25. This material is available free of charge via the Internet at http://pubs.acs.org.

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