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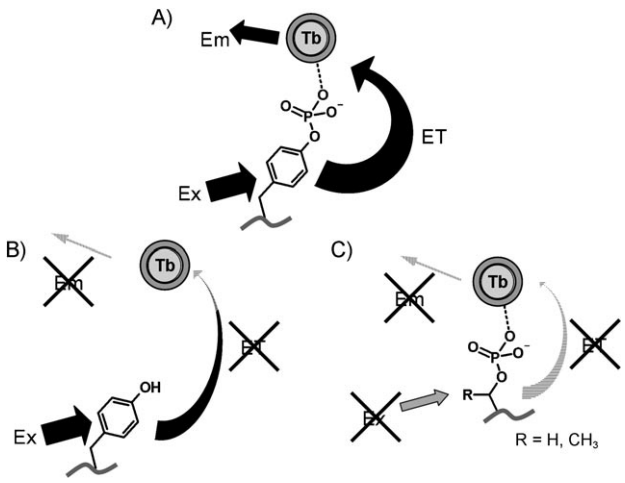
Selective Detection of Phosphotyrosine in the Presence of Various Phosphate-Containing Biomolecules with the Aid of a Terbium(III) Complex

Hiroki Akiba, Jun Sumaoka,* and Makoto Komiyama*[a]

Protein kinases control many cellular processes by specifically phosphorylating serine (Ser), threonine (Thr), or tyrosine (Tyr) residues in their target proteins.[1,2] Among these, the phosphorylation of Tyr is a key step in numerous types of cellular regulation.[2,3] It is well-known that excessive phosphorylation of receptor and nonreceptor tyrosine kinases occurs in some tumor and cancer cell lines.[3] However, phosphorylated Tyr accounts for less than 1% of total phosphorylated amino acids, and many of the roles of phosphotyrosine (pTyr) are not yet clearly known. Accordingly, precise and selective detection of pTyr in vitro and in vivo should allow for deeper understanding of cellular events and their disorders. Simple and stable probes that respond only to pTyr are thus required. Most of the chemical probes used to label phosphoproteins that have been developed over the decades merely work as affinity tags for phosphate groups and hardly discriminate between pTyr and phosphoserine or phosphothreonine (pSer/pThr).[4,5] In this study, pTyr is selectively detected through the emission from a Tb^{III} complex. On interaction of pTyr with the complex, the luminescence from the complex is enormously increased, due to efficient energy transfer from the pTyr benzene ring to the Tb^{III}. In contrast, the other two phosphorylated amino acids (pSer/pThr) are almost silent in emission, although they are also bound by the complex. Clear-cut detection of pTyr is accomplished.

Luminescence from lanthanide ions shows various features such as long lifetimes, large Stokes' shifts, and sharply-spiked emission bands. However, the emission intensity produced by the direct excitation of these ions themselves is relatively small because the f–f transitions are Laporte-forbidden. In order to obtain strong emission, a sensitizing chromophore ("antenna") must be placed close to the ion. Once the antenna absorbs light energy, the ion is excited through an energy transfer process and its luminescence is notably enhanced. Because of this characteristic sensitizing process, both lanthanide ions and their complexes have been used as sensing probes.[6–12] In the case of pTyr detection, Niedbalski et al. reported that luminescence from the Tb^{III} ion is promoted by interaction with pTyr but little affected by other amino acids, including pSer/pThr.[10] However, the Tb^{III} ion also bound other molecules—guanosine-5′-monophosphate (GMP), for example—and emitted notable luminescence (see below). Parker et al. have also developed lanthanide complexes that selectively bind pTyr and further carry antenna moieties in their ligands for intramolecular energy transfer.[11] Considerable selectivity for pTyr detection was accomplished through sophisticated design of the ligand. In terms of the difference in spectral change, mainly based on affinity preference, however, completely clear-cut discrimination was not very easy.[12]

Here we have employed an entirely different strategy for selective detection of pTyr. As depicted in Scheme 1, the Tb^{III} complex used never carries a chromophore as an antenna. 1) When pTyr binds to this Tb^{III} complex through its phosphate group, the distance between the Tb^{III} and the pTyr benzene ring (antenna) is short enough to allow facile energy transfer, and accordingly, the emission from Tb^{III} significantly increases. 2) In contrast, nonphosphorylated Tyr does not have a phosphate group to bind to the Tb^{III}, 3) whereas pSer/pThr has no aromatic ring to work as an antenna. Therefore, neither nonphosphorylated Tyr nor pSer/pThr induces a significant signal, so pTyr is selectively and clearly detected. Nonselective emission from other molecules likely to be present (GMP and nucleic acids) is efficiently suppressed by use of 2,2′,2″,2‴-(1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrayl)tetraacetamide (DOTAM) as the ligand.



Scheme 1. Design of a phosphotyrosine-specific sensor based on the "antenna effect". A) Chromophore-free Tb^{III} complex clearly reveals pTyr through emission from the Tb^{III} ion because the excited energy of the benzene ring of the pTyr is efficiently transferred to the ion. B) Nonphosphorylated tyrosine does not bind to the Tb^{III} complex and efficient energy transfer does not occur, so the emission intensity is not enhanced. C) pSer/pThr does not have a benzene ring to absorb the energy, and so emission intensity is not enhanced. Abbreviations; Ex: excitation; ET: energy transfer; Em: emission.

[a] *H. Akiba, Dr. J. Sumaoka, Prof. Dr. M. Komiyama*
Research Center for Advanced Science and Technology
The University of Tokyo
4-6-1 Komaba, Meguro-Ku, Tokyo, 153-8904 (Japan)
Fax: (+81)3-5452-5209;
E-mail: sumaoka@mkomi.rcast.u-tokyo.ac.jp
komiyama@mkomi.rcast.u-tokyo.ac.jp

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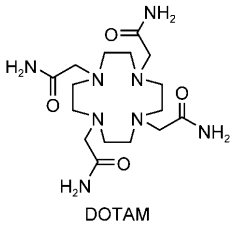


DOTAM

The Tb^{III}•DOTAM complex was prepared as described in the literature.[13] The luminescence from Tb^{III} was detected with excitation at 262.5 nm, at which the benzene ring of pTyr has a strong absorption. Table 1 shows the luminescence intensities (545 nm) from this Tb^{III} complex in the presence of various additives. Both pTyr and phenyl phosphate (model compounds for Tyr-phosphorylated protein) notably increased the luminescence intensity (see also Figure 1 A). These results indicate that the phosphate residues of these compounds interact with the Tb^{III}•DOTAM complex and that the excitation energy of the benzene ring is efficiently transferred to the emitting metal center. On the other hand, Tyr, pSer, and pThr (as well as phenol, Ser, and Thr) induced only marginal increases. Selective sensing of pTyr and phenyl phosphate with the aid of this Tb^{III} complex was therefore successful.

Table 1. Relative enhancement effects of various additives on the luminescence (λ_{em} = 545 nm) from the Tb^{III}•DOTAM complex.

Additive	Luminescence (relative intensity)	Additive	Luminescence (relative intensity)
–[a]	1		
phenol	0.99	phenyl phosphate	9.9
Ser	1.0	pSer	1.0
Thr	1.1	pThr	1.0
Tyr	1.1	pTyr	5.5

[a] Luminescence intensity in the absence of any additive.

We further analyzed the mode of interaction by estimating the *q* value (the number of water molecules coordinated to Tb^{III} ions).[14] Luminescence lifetime measurements indicated $q \sim 1$ for the Tb^{III}•DOTAM complex, whether or not phenyl phosphate is bound (see the Supporting Information for details). Accordingly, no removal of coordinated water occurs and the interaction between Tb^{III}•DOTAM and phenyl phosphate is believed to be ion-pairing rather than direct coordination.[15] The increase in luminescence upon addition of phenyl phosphate is the result of the energy transfer from the benzene ring, rather than the removal of water molecules to quench luminescence.

It is also noteworthy that GMP hardly increased the luminescence intensity from the Tb^{III}•DOTAM (the green line in Figure 1 A). When Tb^{III} ion itself was used, pTyr enhanced the luminescence from the ion, as previously reported by Niedbalski et al. In that case, however, GMP enormously enhanced the luminescence (see the green line in Figure 1 B), as also described in the paper.[10] In the absence of DOTAM, GMP strongly binds to the Tb^{III} ion through multiple coordination both of the phosphate residue and of the guanine ring (N7 and O6; Scheme 2 B).[7a] In this structure, the guanine group is located near the Tb^{III}, so its strong antenna effect gives rise to strong emission from the Tb^{III}. For selective detection of pTyr, background signals of this type have to be suppressed. On the

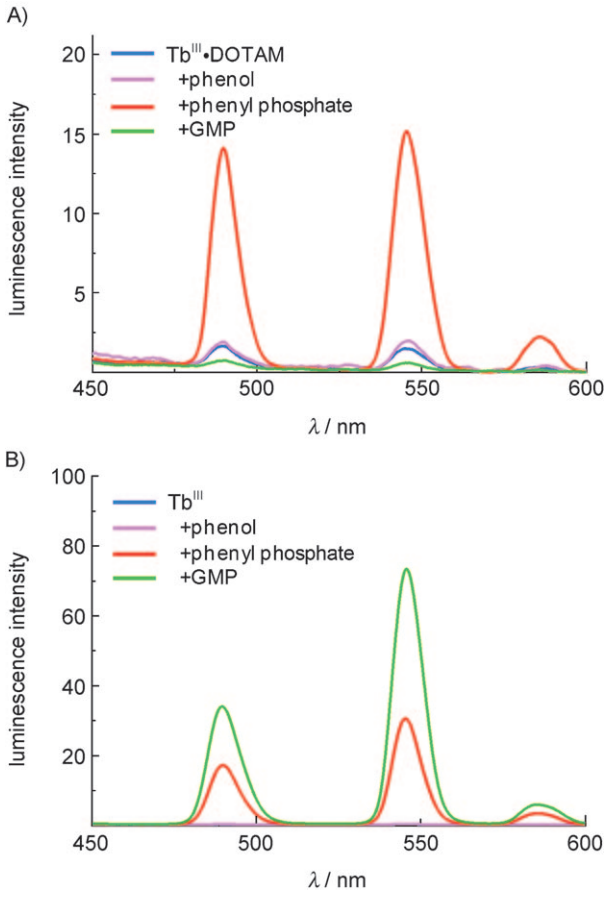
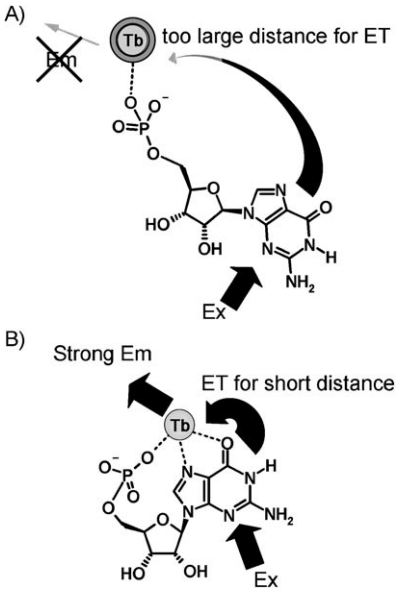


Figure 1. Effects of phenol (magenta), phenyl phosphate (red), and GMP (green) on the emission from A) the Tb^{III}•DOTAM complex, and B) the Tb^{III} ion. Blue lines show the luminescence in the absence of additive. In (B), the blue line and the magenta line are completely superposed.



Scheme 2. Coordination of GMP and enhancement of luminescence emission. A) In the presence of the Tb^{III}•DOTAM complex, the guanine ring is located far away from the Tb^{III} even when its phosphate is bound to the Tb^{III}, so GMP induces a minimal increase in the emission intensity. B) In the case of free Tb^{III} ion, both the phosphate group and the guanine ring in GMP cooperatively bind to the ion and the close proximity induces efficient energy transfer, so strong emission occurs upon addition of GMP.

other hand, Tb^{III}·DOTAM was not sensitized by GMP. This indicates that DOTAM ligand prevents simultaneous interactions by both the phosphate residue and the guanine ring of GMP, so energy transfer is never efficient (Scheme 2A). Similarly, Tb^{III} was sensitized by using single-stranded DNA or RNA,[7] but the Tb^{III}·DOTAM complex was not (see below). Therefore, in order to suppress these background signals, the DOTAM ligand is necessary.

In order to demonstrate further utility of the Tb^{III}·DOTAM complex, the phosphorylation of the Tyr residue in a nonapeptide (Glu-Glu-Glu-Ile-*Tyr*-Glu-Glu-Phe-Asp) was assayed with the aid of this complex. (This oligopeptide is a consensus sequence that is post-translationally modified by *v*-Src on Tyr in cells).[16] The presence or the absence of phosphorylation was clearly distinguishable by the naked eye (compare the two cuvettes photographed in Figure 2A; the spectra are also shown in Figure 2C). When the nonapeptide was not phosphorylated, the luminescence from Tb^{III}·DOTAM was very weak. Upon phosphorylation of its Tyr unit, however, the luminescence was considerably enhanced.[17] Although these sample solutions contained RNA, which is abundant in cytoplasm, its antenna effect never enhanced the emission.[18] In contrast, aqueous solutions of Tb^{III} ion-containing RNA emitted notable luminescence due to the "antenna effect" of the RNA, even when the nonapeptide was not phosphorylated (Figure 2B, left). Accordingly, discrimination between phosphorylated and nonphosphorylated peptides was relatively difficult (Figure 2B and 2D). The use of the Tb^{III}·DOTAM complex is absolutely necessary to assay these specimens. Attachment of recognition moieties to the complex should further enhance the selectivity.

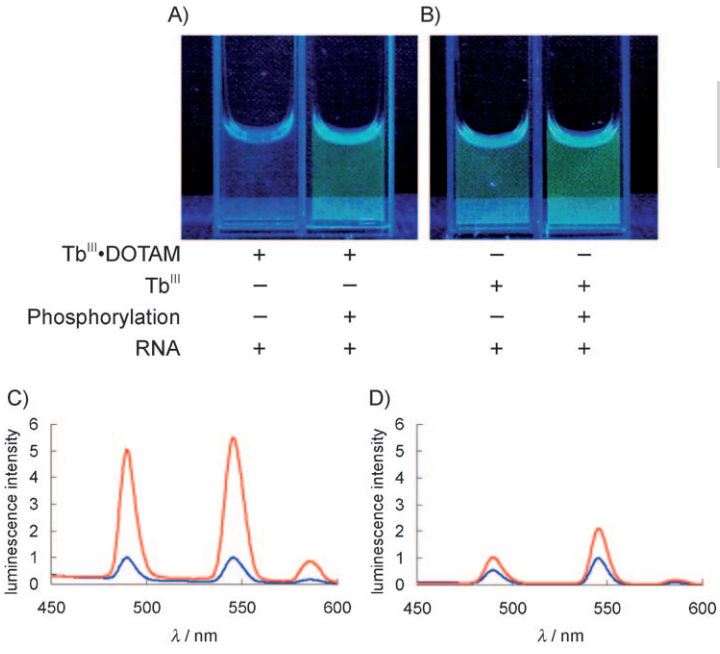


Figure 2. The detection of Tyr phosphorylation on the *v*-Src substrate nonapeptide EEEIYEEFD in RNA-containing solutions. A) and B) are photographs of solutions of A) the Tb^{III}·DOTAM complex (100 μM) and B) the Tb^{III} ion (20 μM) containing either nonphosphorylated peptide (left) or its phosphorylated product (right). The RNA (1/200 equivalent to the oligopeptide) was added as a typical biomolecule that might also be present in the sample solutions. A handheld UV lamp (λ_{ex} = 254 nm) was used as a light source. C) and D) are luminescence spectra corresponding to A) and B), respectively. Red line: phosphopeptide; blue line: nonphosphorylated peptide. The spectra are normalized so that the luminescence intensity at 545 nm with the addition of nonphosphorylated peptide is 1.

In conclusion, the Tb^{III}·DOTAM complex provides a simple and robust method for selective detection of phosphorylation of tyrosine in peptides. Applications of this finding to various analyses, as well as further optimization of the ligand structure, are currently underway in our laboratory.

Experimental Section

Luminescence measurements were conducted in 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer (pH 7.0, 10 mM) containing either Tb^{III}·DOTAM complex or Tb^{III} ion (100 μM) unless otherwise stated. Additives were added to the solution to a concentration of 100 μM. The spectra were measured with a FP-6500 fluorimeter (JASCO, Tokyo, Japan) with excitation at 262.5 nm, soon after the additives had been added.

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Keywords: lanthanides · luminescence · phosphopeptides · terbium · tyrosine

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[17] The Tb^{III}·DOTAM luminescence spectrum in the presence of this peptide is shown in Figure S3 in the Supporting Information. This peptide has many negative charges that electrostatically interact with positively charged Tb^{III}·DOTAM.

[18] The sequence of this RNA is UGU UCA CAA CGA CUU UCG CGU CGG UGG GGU UGG CUU CAA A. Of 40 nucleobases, twelve bases are guanine.

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