

A Fluorescent Reporter of the Phosphorylation Status of the Substrate Protein STAT3**

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The ability to monitor phosphorylation events can provide valuable information pertaining to signal transduction regulation, and for developing effective therapeutics targeted at aberrant kinase activities.^[1] Kinase activity can be optically reported by using sensors based on short peptides or domains that resemble amino acids near the phosphorylation site of a substrate protein. Of these sensors, peptide-based reporters exhibit large fluorescence changes from small-molecule fluorophores that are chemically attached to the peptide sensor,^[2] but the introduction of these reporters into cells is challenging. Protein-based reporters contain genetically appended fluorescent proteins and have revealed novel spatiotemporal information regarding kinases in living cells despite the moderate signal changes of these reporters.^[3] Nonetheless, the subcellular location, trafficking, and lifetime of a full-length substrate protein cannot be faithfully replicated by such comparatively simplified peptide or domain sensor elements.^[4] Moreover, many kinases derive substrate specificity by using distal residues in addition to those proximal to the phosphorylation site, and can have multiple substrate proteins.^[5] Therefore, current kinase reporters provide limited information on the phosphorylation state of a particular substrate protein.

Herein we present a method to optically report the phosphorylation status of a specific full-length substrate protein, signal transducer, and activator of transcription 3 (STAT3), which plays a leading role in many oncogenic and developmental pathways.^[6] A small-molecule fluorophore in

the format of an unnatural amino acid (Uaa) was genetically introduced into STAT3 to sense its phosphorylation state. A large fluorescence change was observed when the STAT3 probe was phosphorylated by Src kinase *in vitro* and when it was incubated with endogenously activated STAT3 from mammalian nuclear extracts. This method enables optical investigation of protein phosphorylation on the substrate level with high specificity.

Our strategy is to genetically incorporate a fluorescent Uaa into the target protein at a site close to the residue subject to phosphorylation (Figure 1a). The negatively

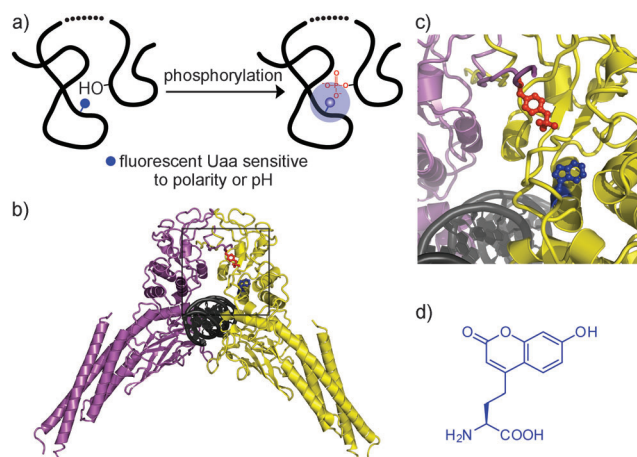


Figure 1. Reporting the phosphorylation status of a substrate protein using genetically encoded Uaas. a) Schematic illustration. The broken line indicates that the phosphorylated residue and the fluorescent Uaa can be on the same or separate proteins. b) Crystal structure of the STAT3 β homodimer (PDB 1BG1) depicting two monomers (pink and yellow) binding DNA (gray). c) Region framed in (b) illustrating the location of pTyr705 (red) in relation to Trp564 (blue). d) Structure of L-(7-hydroxycoumarin-4-yl) ethylglycine (7HC).

charged phosphate group may alter the local polarity or pH value, to which the fluorophore of the Uaa is designed to be sensitive. By using a full-length substrate protein, one can incorporate the fluorescent Uaa at any site close to the phosphorylated residue in the tertiary structure, providing more flexibility in choosing the optimal sensor location than in peptide-based methods. The closely positioned phosphorylated residue and the fluorescent Uaa can be within the same target protein or in different proteins if the target protein is oligomeric or part of a complex.

Upon phosphorylation on Tyr705, STAT3 dimerizes through the reciprocal binding of phosphotyrosine (pTyr)

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into the SH2 domain of an opposing monomer (Figure 1 b). The dimer subsequently translocates into the nucleus as an activated transcription factor. We reasoned that introduction of the negatively charged pTyr705 into the SH2 domain would alter the pH value within the binding pocket and that a pH-sensitive fluorophore should report such a change. A good candidate is 7-hydroxycoumarin (quantum yield = 0.63), whose fluorescence intensity and excitation wavelength are pH-dependent with a pKa of approximately 7.8.^[7] Based on the crystal structure of the DNA-bound STAT3 β homodimer (Figure 1 b),^[8] we selected Trp564 for mutation to L-(7-hydroxycoumarin-4-yl) ethylglycine (7HC, Figure 1 d). Trp564 is located within the second layer of the SH2 binding pocket close to the pTyr of the opposing monomer, but distant from Tyr705 of the same monomer and outside of the DNA binding domain (Figure 1 c). Trp is also similar in size to 7HC. Collectively, these properties should minimize any potential interference from introducing 7HC.

7HC was genetically incorporated into the STAT3 β isoform in *E. coli* by using a reported orthogonal tRNA/aminoacyl-tRNA synthetase pair^[9] to suppress the 564TAG codon in our optimized expression system (see the Supporting Information). To verify 7HC incorporation, cell lysates were analyzed by Western blot by using an antibody against STAT3 (Figure 2 a). Full-length STAT3 β was observed only when 7HC was added to the growth medium. Wild type (wt) and

7HC-containing STAT3 β proteins (7HC-STAT3 β) were purified with nickel-nitrilotriacetic acid (Ni-NTA) chromatography. A single bright blue fluorescent band was observed only for purified 7HC-STAT3 β on SDS-PAGE (Figure 2 b and Figure S1 in the Supporting Information). Incorporation of 7HC into STAT3 β at the TAG site was confirmed by using MS (Figure S2).

To determine if Trp564 mutation to 7HC affects STAT3 function, purified 7HC-STAT3 β protein was tested in vitro for its ability to be phosphorylated by the nonreceptor tyrosine kinase Src and its ability to bind the high-affinity sis-inducible element (hSIE) consensus DNA sequence in an electrophoretic mobility shift assay (EMSA). After incubation with Src, samples were separated by SDS-PAGE and transferred onto a blot, which was probed with a STAT3 antibody specific for pY705 (Figure 2 c). A clear band at the same molecular weight was seen for both wt STAT3 β and 7HC-STAT3 β only when phosphorylated. In the EMSA, the ³²P-labeled DNA probe shifted to the same position for both phosphorylated wt STAT3 β and 7HC-STAT3 β (Figure 2 d). When excess nonradiolabeled hSIE probe was introduced into EMSA binding reactions, specific competition was seen for both 7HC-STAT3 β and wt STAT3 β . The relative affinities for hSIE, as quantified by K_d , were almost identical. These results indicate that 7HC-STAT3 β can be phosphorylated and bind a consensus DNA sequence with similar affinity to wt STAT3 β , thereby suggesting that substitution of Trp564 with 7HC does not alter STAT3 function.

We next tested if the 7HC could sense and report the phosphorylation of STAT3 β by using fluorometry (Figure 3 a). Before phosphorylation, 7HC-STAT3 β exhibited very weak fluorescence with a single emission peak at 448 nm. After incubation with Src kinase, the fluorescence

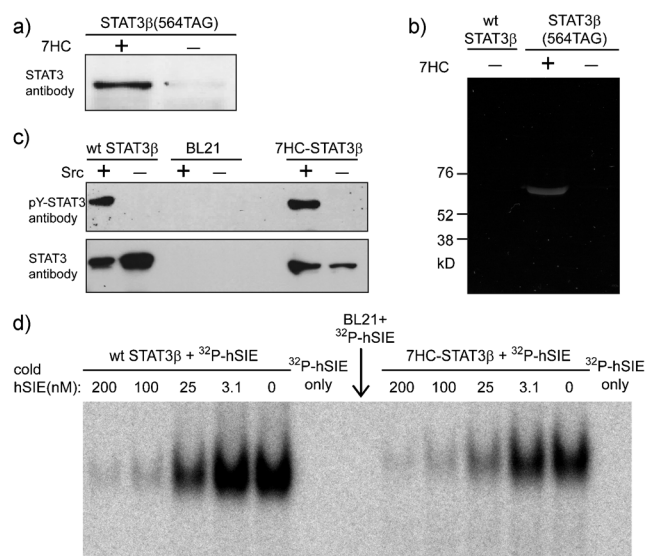


Figure 2. Similar to wt STAT3 β , 7HC-STAT3 β can be phosphorylated and binds a consensus DNA sequence. a) Western blot analysis of *E. coli* lysates from cells expressing STAT3 β (564TAG) and the 7HC-specific tRNA/synthetase pair. b) Photograph of SDS-PAGE analysis of wt STAT3 β and 7HC-STAT3 β (564TAG) expressed in the presence and absence of 7HC. The gel was exposed to 365 nm UV light. c) Western blot of protein samples incubated with and without Src kinase. Probing with a STAT3-specific antibody ensured that comparable amounts of STAT3 were loaded. d) EMSA using ³²P-labeled hSIE DNA probe. Bands for the probe incubated with wt STAT3 β and 7HC-STAT3 β were upward-shifted, indicating that both proteins bind the hSIE probe. Specific competition was seen with excess unlabeled probe yielding dissociation constants for wt STAT3 β ($K_d = (6.3 \pm 0.6)$ nM, $n = 4$) and 7HC-STAT3 β ($K_d = (6.8 \pm 1.6)$ nM, $n = 4$). n = number of independent binding reactions using different quantities of STAT3 protein.

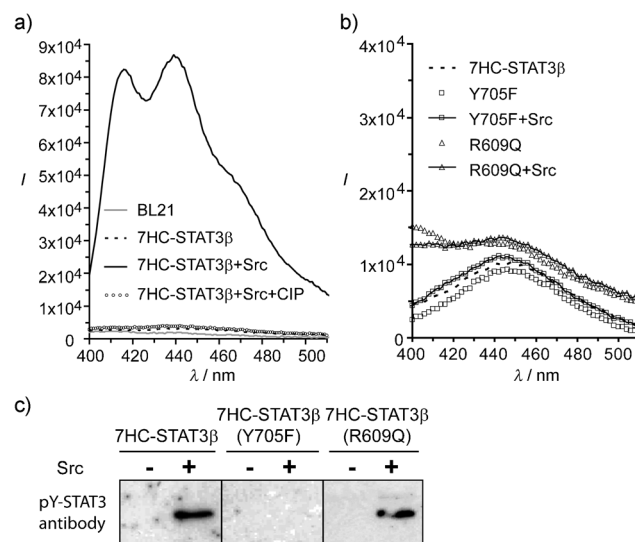


Figure 3. 7HC-STAT3 β reversibly reports the phosphorylation status of STAT3. a) Fluorescence emission of 7HC-STAT3 β before and after phosphorylation by Src kinase followed by dephosphorylation by CIP. b) Fluorescence emission of 7HC-STAT3 β mutants Y705F and R609Q before and after phosphorylation by Src kinase. c) Western blots for 7HC-STAT3 β and mutants using an antibody against phosphorylated STAT3. Equivalent amounts of STAT3 protein were loaded in each lane.

intensity of 7HC-STAT3 β increased markedly. A 13-fold increase (13 ± 4.3 , $n=6$) was detected for 20 nM 7HC-STAT3 β , suggesting that the reporter is highly sensitive. In addition, a second emission peak emerged at 416 nm. When calf intestinal phosphatase (CIP) was added, the fluorescence intensity dropped back to the level of unphosphorylated 7HC-STAT3 β , indicating that the fluorescence change is reversible and dependent on phosphorylation status.

To confirm that the observed fluorescence change in 7HC-STAT3 β was specifically due to phosphorylation of Tyr705, we introduced a Y705F mutation, which does not allow STAT3 phosphorylation at Tyr705^[10] (Figure 3c). This mutant had the same fluorescence emission spectrum as 7HC-STAT3 β , but showed no fluorescence change upon incubation with Src (Figure 3b). We made another mutation, R609Q, which prevents binding of pTyr705 into the SH2 domain.^[11] The 7HC-STAT3 β R609Q mutant could still be phosphorylated by Src kinase (Figure 3c), yet exhibited no fluorescence change (Figure 3b). Collectively, these results indicate that the observed fluorescence change can be attributed to the phosphorylation of Tyr705 that subsequently binds to the SH2 pocket containing 7HC.

To understand the sensing mechanism, we measured the fluorescence spectra of 7HC at different pH values in aqueous buffer (Figure 4a). Consistent with 7-hydroxycoumarin,^[7] at low pH values, 7HC showed an excitation peak at 325 nm that corresponds to the neutral phenol form, and, at high pH values, a peak at 365 nm that corresponds to the anionic phenolate form. Consistent with the pH-induced shift of 7HC,

the excitation peak for 7HC-STAT3 β shifted from 325 nm to 365 nm upon phosphorylation (Figure 4c). In addition, when excited at a wavelength longer than the isosbestic point (335 nm), the emission intensity of 7HC increased with the pH value (Figure 4b) because of the higher concentration of the phenolate species in the ground state. By using a similar excitation wavelength to 7HC, the fluorescence intensity of 7HC-STAT3 β increased after phosphorylation, thus further implying a local pH value increase. Both the shifted excitation peak and increased emission intensity of 7HC-STAT3 β consistently suggest that the pH value within the 7HC microenvironment increased upon phosphorylation. This pH value increase results in deprotonation of phenolic 7HC in 7HC-STAT3 β to the phenolate form, which may arise from an altered local hydrogen-bonding network induced by the incoming phosphate group. Moreover, crystal structures of the unphosphorylated and phosphorylated STAT3 protein show almost no conformational change after phosphorylation of Tyr705,^[12] suggesting that a conformational change upon pTyr705 binding to the SH2 domain is not responsible for the observed 7HC fluorescence change.

Another unique spectroscopic feature of 7HC-STAT3 β is the appearance of an emission peak at 416 nm after phosphorylation; this peak provides a characteristic readout that has not been reported for other proteins that contain 7HC.^[9] This emission peak corresponds to the excited state of the neutral phenol form of 7HC.^[13] When 7-hydroxycoumarin is excited in aqueous solution above pH 2, only a single emission peak at 456 nm, which corresponds to the excited phenolate species, is observed, regardless of which species is excited.^[7] We observed the same result for 7HC in aqueous buffer (Figure 4b). This behavior is due to rapid deprotonation of the neutral phenol form of 7-hydroxycoumarin in the excited state, which occurs within the lifetime of the singlet excited state in aqueous solution.^[7] When 7-hydroxycoumarin is excited in H₂O mixed with solvents that are less efficient proton acceptors than H₂O, the intensity of the emission peak corresponding to the excited neutral phenol form of 7-hydroxycoumarin increases as the mole fraction of H₂O decreases.^[14] This emission thus indicates a reduced accessibility of the fluorophore to H₂O. In the 7HC-STAT3 β protein, a single emission peak corresponding to the phenolate form was observed before phosphorylation (Figure 3a,b), signifying the accessibility of 7HC to water and very rapid excited-state deprotonation. The additional emission peak at 416 nm corresponding to the neutral phenol form of 7HC emerged only after phosphorylation (Figure 3a). This observation indicates that deprotonation of the phenol form at the excited state was no longer rapid and that 7HC became shielded from water, possibly due to pTyr705 and its neighboring residues filling the SH2 pocket.

To test if 7HC-STAT3 β can report the phosphorylation status of STAT3 proteins in mammalian cellular media, we incubated 7HC-STAT3 β with nuclear extracts from human hepatoma HepG2 cells. The cytokine interleukin-6 (IL-6) is a potent physiological activator of STAT3. Upon binding of IL-6 to its cytokine receptor, STAT3 is phosphorylated at Tyr705 by the receptor-associated and activated Janus kinase, and then translocates into the nucleus. Consistent with a previous

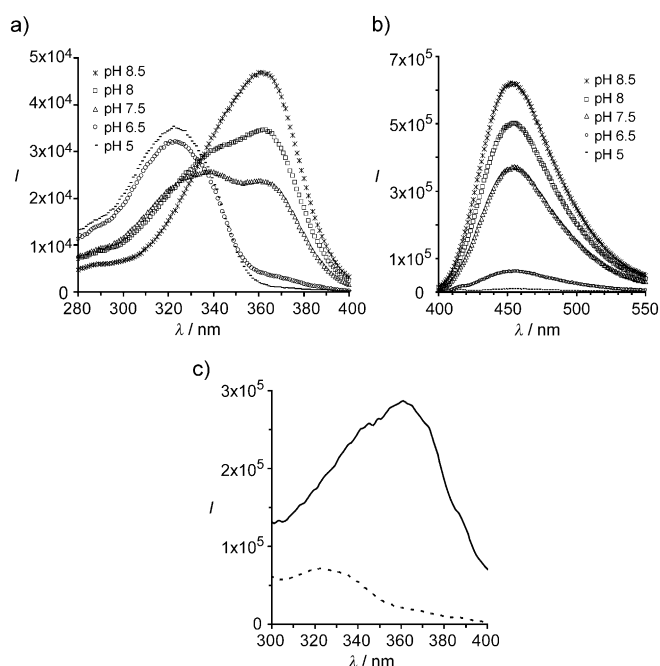


Figure 4. 7HC in the 7HC-STAT3 β protein experiences a pH change upon phosphorylation. a) Fluorescence excitation spectra of 7HC in aqueous buffer with emission recorded at 450 nm. b) Fluorescence emission spectra of 7HC in aqueous buffer with excitation at 363 nm. c) Fluorescence excitation spectra of 7HC-STAT3 β with emission recorded at 450 nm before (-----) and after (—) phosphorylation by Src kinase.

report,^[15] we detected a high level of phosphorylated STAT3 in the nucleus of HepG2 cells only when treated with IL-6 (Figure 5a). We incubated the same amount of 7HC-STAT3 β with these nuclear extracts and found that the fluorescence intensity increased only slightly (1.4-fold) for those from

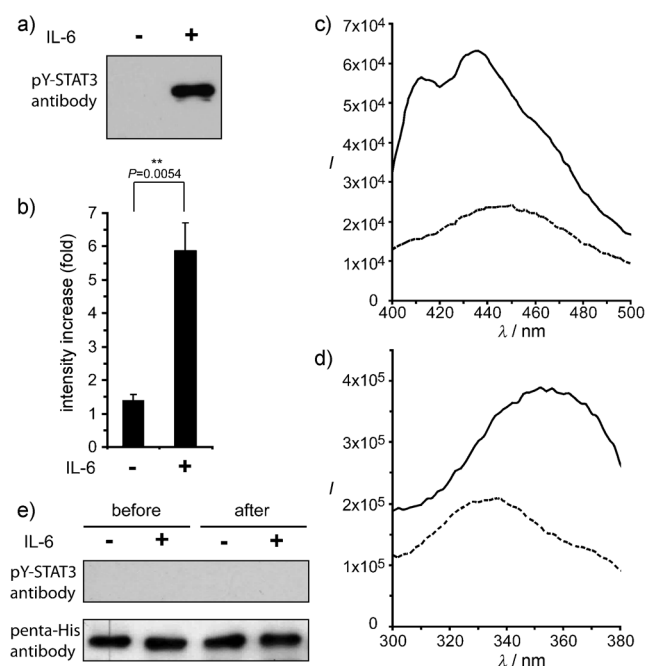


Figure 5. 7HC-STAT3 β reports the phosphorylation status of endogenous STAT3 from HepG2 cells. a) Western blot showing that STAT3 was phosphorylated in the nucleus of HepG2 cells only when activated by IL-6. b) Fluorescence intensity increase of 7HC-STAT3 β upon incubation with nuclear extracts. The values ($\pm$ SEM) were: without IL-6 (–) 1.4 ± 0.2 and with IL-6 (+) 5.9 ± 0.8 , $n=3$ from three independent batches of cells. The IL-6 activated nuclear fraction was statistically different from the uninduced sample (Student's t-test, two-tailed, unpaired). c) Fluorescence emission spectra and d) excitation spectra of 7HC-STAT3 β after incubation with the nuclear extracts of HepG2 cells with (—) and without (---) IL-6 induction. e) Western blot showing that 7HC-STAT3 β was not phosphorylated by cell lysates. 7HC-STAT3 β mixed with cell lysates before ($t=0$) and after ($t=2$ h) incubation were analyzed. 7HC-STAT3 β was N-terminally truncated and thus ran at a different position from endogenous STAT3. The blot was also probed with the penta-His antibody to detect the C-terminal His6 tag appended on 7HC-STAT3 β .

uninduced cells but significantly (5.9-fold) for those treated with IL-6 (Figure 5b). This result indicates that 7HC-STAT3 β can indeed optically report the phosphorylation status of endogenous STAT3.

To understand the observed difference, we analyzed the excitation and emission spectra of the nuclear extract samples after incubation with 7HC-STAT3 β (Figure 5c,d). The nuclear fraction of IL-6 induced cells showed the red-shifted excitation peak and double emission peaks characteristic of 7HC-STAT3 β phosphorylated by Src (Figures 4c and 3a), whereas uninduced nuclear fractions showed the same excitation and emission spectra as unphosphorylated 7HC-STAT3 β . These results indicate that only in the nuclear

fraction of IL-6 induced cells did binding of 7HC-STAT3 β to pTyr705 occur. Three possibilities can lead to such binding: 1) 7HC-STAT3 β is phosphorylated by endogenous kinases in the nuclear extract, after which it forms a homodimer or a heterodimer with endogenous phosphorylated STAT3; 2) another phosphoprotein binds the SH2 domain of 7HC-STAT3 β ; 3) unphosphorylated 7HC-STAT3 β forms a heterodimer with phosphorylated endogenous STAT3. To examine the first possibility, an antiphosphotyrosine STAT3 antibody was used to probe 7HC-STAT3 β incubated in the nuclear lysate samples. Phosphorylation of 7HC-STAT3 β was not detected in samples with or without IL-6 induction (Figure 5e). No other phosphoproteins, except other STAT molecules, have been reported to bind the SH2 domain of pSTAT3 with high affinity, but we are nevertheless planning cross-linking experiments to determine whether additional proteins could compete with STAT3 in forming homodimers. In addition, it is known that STAT3 α and STAT3 β isoforms can form homodimers and heterodimers with each other.^[16] We therefore favor the conclusion that after being added to the nuclear fraction of IL-6 induced cells, 7HC-STAT3 β is not phosphorylated but forms a heterodimer with endogenous phosphorylated STAT3 protein, resulting in the expected fluorescence intensity increase, characteristic double emission peaks, and excitation peak shift.

In summary, we developed a fluorescent reporter for the phosphorylation status of STAT3 by genetically incorporating the fluorescent Uaa 7HC into a selected site in STAT3 β . As Trp564 is conserved in all seven mammalian STAT proteins,^[8] this method should be transferable to detect the phosphorylation of other STATs, which will be valuable to untangle the function of different STATs and various STAT isoforms selectively. A similar strategy could be applied to other SH2 domain-containing proteins, which participate in a variety of signal transduction pathways. A reporter based on the full-length substrate protein represents cellular characteristics of the target protein with high fidelity, and can be used to report kinase as well as phosphatase activity with high specificity. Toward the goal of expanding this method into mammalian cells, we are currently evolving an orthogonal tRNA-synthetase pair that will enable the genetic incorporation of 7HC into proteins in live mammalian cells.

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