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Semisynthesis of unnatural amino acid mutants of paxillin: Protein probes for cell migration studies

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Abstract

Caged phosphopeptides and phosphoproteins are valuable tools for dissecting the dynamic role of phosphorylation in complex signaling networks with temporal and spatial control. Demonstrating the broad scope of phosphoamino acid caging for studying signaling events, we report here the semisynthesis of a photolabile precursor to the cellular migration protein paxillin, which is a complex, multidomain phosphoprotein. This semisynthetic construct provides a powerful probe for investigating the influence that phosphorylation of paxillin at a single site has on cellular migration. The 61-kDa paxillin construct was assembled using native chemical ligation to install a caged phosphotyrosine residue at position 31 of the 557-residue protein, and the probe includes all other binding and localization determinants in the paxillin macromolecule, which are essential for creating a native environment to investigate phosphorylation. Following semisynthesis, paxillin variants were characterized through detailed biochemical analyses and by quantitative uncaging studies.

Keywords: paxillin; native chemical ligation; caging; phosphoprotein; phosphorylation; semisynthesis

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The control of cellular adhesion and migration is essential for the regulation of biological processes, including embryogenesis, wound repair, and metastasis (Lauffenburger and Horwitz 1996). Paxillin is a multidomain protein that orchestrates a pivotal role in these processes by acting as a dynamic scaffold for signaling and structural proteins (Turner 2000). The phosphorylation of paxillin at specific residues spanning the macromolecule creates distinct protein-binding sites and thereby directs paxillin localization to focal adhesions, sites of cell contact with the extracellular matrix, and influences the controlled assembly and dissolution of signaling cascades (Brown and Turner 2004). For investigating processes such as cell migration, there is a need for tools that enable researchers to dissect the dynamic roles of protein phosphorylation within complex signaling networks.

The essentiality of specific protein phosphorylation events can be assessed by a number of approaches, including gene knockout, RNA interference, and site-directed mutagenesis. One limitation with these strategies is that they cannot afford information on phosphorylation in ''real time.'' As a complement to these approaches, the synthesis of caged phosphopeptides, which enable the controlled release of specific phosphorylated species upon photolysis, was introduced (Rothman et al. 2002). Recently, a general method for the synthesis of these probes has facilitated the application of caged phosphopeptides in cellular studies (Nguyen et al. 2004; Humphrey et al. 2005). Expanding on this work, and as part of an initiative to develop generalizable approaches for the preparation of full-length caged phosphoproteins, we report the

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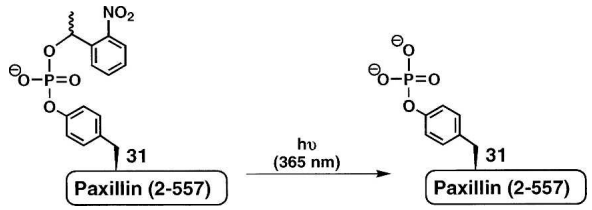

Figure 1. Caged phosphotyrosine 31 paxillin. Uncaging with long-wavelength UV light releases phosphotyrosine 31 paxillin.

semisynthesis and biochemical characterization of a mutant of the 61-kDa protein paxillin that includes a caged phosphotyrosine (cpTyr) residue at position 31 within the N terminus (Fig. 1). We similarly report related paxillin variants with a Tyr or phosphotyrosine (pTyr) at residue 31, which were constructed through a parallel approach to furnish the nonphosphorylated and discretely phosphorylated species as biological controls.

The semisynthesis of the three paxillin variants was accomplished using native chemical ligation (NCL). NCL is a chemoselective technique that enables a synthetic peptide to be joined to a second peptide or a biologically expressed protein fragment through a native peptide bond (Dawson et al. 1994; Muir 2003). Required for this reaction are a C-terminal thioester on one moiety and a free cysteine residue at the N terminus of the second moiety. There are several examples in which NCL has been applied to create probes for the study of protein phosphorylation. These include the semisynthesis of phosphatase-resistant phosphoprotein analogs (Lu et al. 2003; Zheng et al. 2005) and a dually caged phosphoSer-containing derivative of a domain of the protein Smad2 (Hahn and Muir 2004). With the semisynthesis of paxillin analogs, we demonstrate that NCL can also be applied for the assembly of caged and phosphorylated variants of full-length proteins, exemplified by targeting a large, multidomain adaptor protein. Importantly, the paxillin probe comprises the entire paxillin macromolecule, including all other binding and localization domains and determinants, which are essential for creating a native-like system to investigate phosphorylation at a single site. The approach reported here enables the semisynthesis of a variety of unique paxillin analogs with modifications in the N-terminal domain (residues 2–36) in quantities sufficient for complete biochemical characterization and subsequent use in biological investigations.

Following semisynthesis, the paxillin variants were characterized by in vitro binding to a selection of cognate proteins, by phosphorylation using partner kinases and probed with phosphoprotein-specific antibodies, and by quantitative uncaging studies. The semisynthetic caged phosphoTyr31 paxillin permits the time-sensitive investigation of a single phosphorylation event and will serve as a valuable tool to probe the role of Tyr31-paxillin phosphorylation in cellular migration.

Results and Discussion

For the semisynthesis of paxillin (Y31Pax), phosphoTyr31 paxillin (pY31Pax), and caged phosphoTyr31 paxillin (cpY31Pax), synthetic thioesters corresponding to residues 2–36 of paxillin were ligated to a biologically expressed segment comprising paxillin residues 37–557 (Fig. 2). An Asn37Cys mutation was designed in all constructs to provide a Gly36-Cys37 junction for NCL. This site was selected because it is in a region of paxillin free from predicted secondary structure, and because the presence of a Gly as the terminal thioester residue is known to increase ligation efficiency (Hackeng et al. 1999).

Three peptide thioesters were synthesized corresponding to residues 2–36 of paxillin, and incorporating a Tyr, pTyr, or cpTyr building block at residue 31 (Fig. 2A). The 41-residue peptides (Ac-HHHHHH-DDLDALLADLEST TSHISKRPVFLSEETP-X-SYPTG, X = Tyr [**1a**], pTyr[**1b**], or cpTyr [**1c**]) were prepared by (fluorenylmethoxy)carbonyl (Fmoc)-based solid-phase peptide synthesis (SPPS) on the highly acid-labile TGT resin. The peptides were released from the resin with a C-terminal carboxylic acid and side-chain protection intact, and were subsequently derivatized to afford the corresponding C-terminal thioesters (Futaki et al. 1997; Mezo et al. 2001). The cpTyr building block was synthesized as previously described (Rothman et al. 2003) to install a pTyr masked by the 1-(2-nitrophenyl)-ethyl (NPE) caging group. An N-terminal hexahistidine tag was included in the synthetic peptides to provide a handle for visualization and purification of ligation products after NCL.

For the C-terminal fragment of paxillin, residues 37–557 were expressed as a GST-fusion construct with a FLAG tag (DYKDDDDK) included at the C terminus to provide a second handle for purification (Fig. 2B). A TEV protease cleavage sequence, ENLYFQC, was incorporated immediately preceding the paxillin insert. TEV protease is a highly selective cysteine protease that typically recognizes a serine or glycine in the P1′ site, but also will accept a cysteine residue at that position (Tolbert and Wong 2002). Therefore, treatment of the GST-paxillin(38–557)-FLAG protein (**2**) with TEV protease concurrently removed the GST tag and revealed an N-terminal cysteine residue to afford Cys37-paxillin(38–557)-FLAG (**3**) for subsequent ligation. Paxillin is known to be a challenging protein to express in *Escherichia coli*, in part because of a significant number of rare codons, including 27 rare CCC (Pro) codons. Therefore, to access sufficient quantities of protein and overcome expression and truncation difficulties, the protein was fermented in 10-L batches using codon-enhanced cells and purified via both the N-terminal GST and C-terminal FLAG tags. The FLAG tag was essential for separating any truncation products from the full-length protein.

The ligations between **1a**, **1b**, or **1c** and **3** were conducted in nondenaturing conditions to access Y31Pax

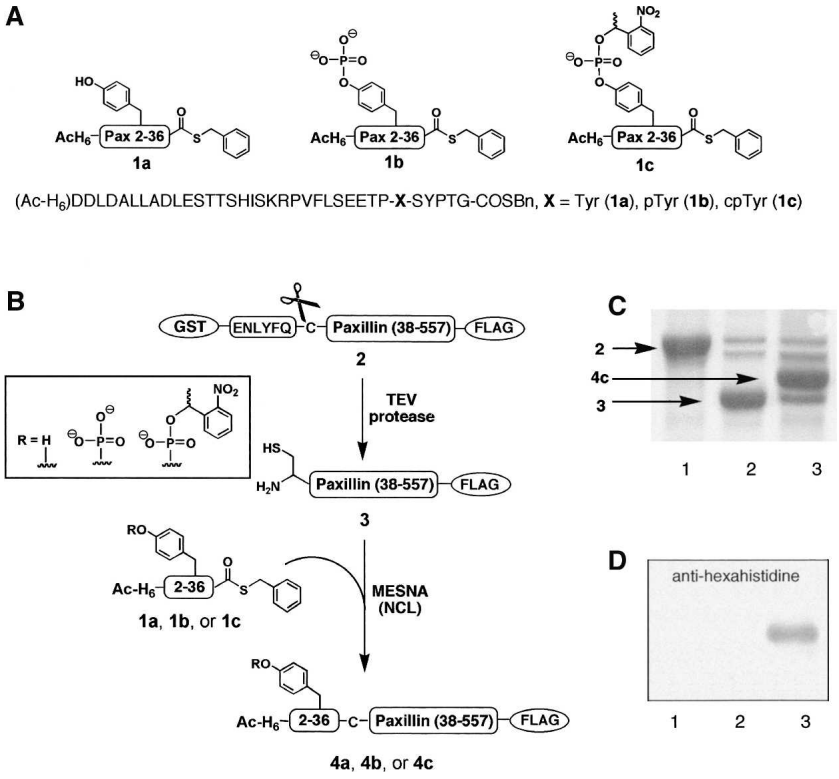

Figure 2. Semisynthesis of paxillin analogs Y31Pax (**4a**), pY31Pax (**4b**), and cpY31Pax (**4c**). (*A*) Synthetic peptide thioesters corresponding to the N terminus of paxillin and incorporating a Tyr (**1a**), pTyr (**1b**), or cpTyr (**1c**) at position 31. (*B*) The semisynthetic strategy. The biologically expressed GST-paxillin(38–557) fusion protein **2** contains N-terminal and C-terminal purification tags to enable isolation from truncation and degradation products. Treatment of **2** with TEV protease results in the generation of the N-terminal cysteine-containing fragment, Cys-paxillin(38–557)-FLAG (**3**). NCL joins fragment **3** with a paxillin thioester to generate **4a**, **4b**, or **4c**. Coomassie (*C*) and hexahistidine-probed (*D*) Western blot analysis of **4c** showing the purified GST-fusion protein GST-paxillin(38–557)-FLAG (**2**) (lane *1*); the TEV cleavage product, Cys-paxillin(38–557)-FLAG (**3**) (lane *2*); and the crude NCL product, cpY31Pax (**4c**) (lane 3). Only the ligated product is visible on the Western blot, since the hexahistidine tag is introduced by the synthetic thioester.

(**4a**), pY31Pax (**4b**), and cpY31Pax (**4c**) in multimilligram quantities (Fig. 2C, D). Exchange of the semisynthetic proteins into PBS using 50-kDa MWCO dialysis membrane concurrently removed unreacted peptide thioester (MWt 4.8–5.1 kDa).

In vitro characterization of semisynthetic paxillin

Since paxillin is a molecular adaptor protein with no known enzymatic activity, the function of the reconstituted paxillin was validated in vitro by analyzing binding to known paxillin-binding partners and by assessing activity with kinases that are documented to phosphorylate paxillin. The paxillin-binding partners focal adhesion kinase (FAK) (Hildebrand et al. 1995), GRK interactor 1 (GIT1) (Manabe et al. 2002), and PTP-PEST (Shen et al. 1998) were selected to probe binding along the entire length of the protein, while the phosphorylation studies were focused on the NCL-introduced N terminus, which includes the Tyr31 site (Fig. 3A).

Binding to selected paxillin-interacting proteins

For binding studies, N-terminal GST tags, which are absent in the ligated paxillin constructs, were expressed with the paxillin-binding regions of FAK, GIT, and PTP-PEST. The three GST-tagged constructs, GST-FAK(857–1057), GST-GIT(622–761), and GST-PTP-PEST(338–390), were evaluated for binding to the semisynthetic Y31Pax (**4a**) in a modified GST pull-down assay (Fig. 3B). The expressed GST-tag alone (27 kDa) was tested as a negative control to assess nonspecific interactions. Paxillin binding was detected strongly with the FAK and GIT constructs, negligibly with GST alone, and weakly with the PTP-PEST construct. Since PTP-PEST binds at the C-terminal zinc-binding LIM domains of paxillin (Shen et al. 1998), we evaluated whether improper folding of these domains due to insufficient zinc in the purified construct contributed to the poor PTP-PEST binding characteristics. The addition of $ZnCl_2$ to the paxillin solution significantly increased PTP-PEST binding. Future samples of paxillin and semisynthetic

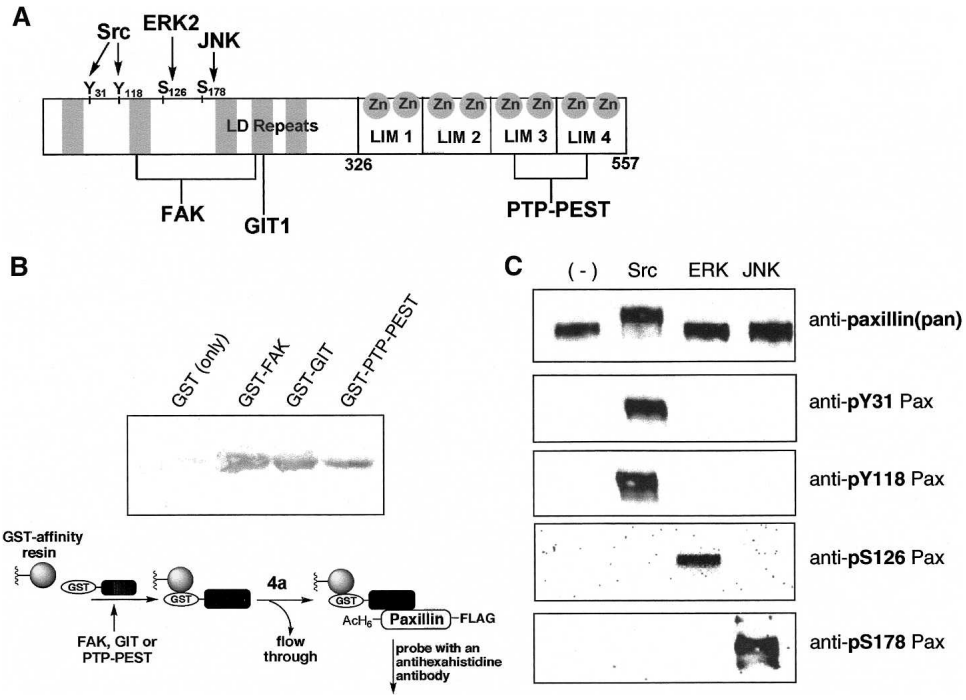

Figure 3. (*A*) Schematic representation of paxillin with selected binding partners (bold lines) and upstream kinases (arrows) to the corresponding phosphorylated residue. (*B*) In vitro binding assay of semisynthetic paxillin (**4a**) with a negative control (GST), and GST-fusions of the paxillin binding regions of FAK, GIT, and PTP-PEST. The GST and GST fusions were bound to solid support, incubated with **4a**, washed, and then probed for paxillin binding with an anti-hexahistidine Western blot. (*C*) Phosphorylation of semisynthetic Y31Pax (**4a**). Paxillin was treated with Src, ERK, JNK, or no kinase in the presence of $MgCl_2$ and ATP, and phosphorylation was visualized by phosphospecific anti-paxillin [pY31], [pY118], [pS126], and [pS128]. The reactions were also probed with a general anti-paxillin (pan) antibody to demonstrate comparable loading. (−) Negative control treated with no kinase.

paxillin derivatives were purified in the presence of 1 mM $ZnCl_2$. These binding experiments suggest that the semisynthetic paxillin construct interacts with known paxillin binding proteins comparably to native paxillin.

Phosphorylation by upstream kinases

Next, to demonstrate that the semisynthetic paxillin analog functions as a substrate for selected kinases that natively phosphorylate the protein, phosphorylation of **4a** was attempted with Src, ERK, and JNK and probed with phosphorylation-specific antibodies to four sites along paxillin (Fig. 3C). Src is a tyrosine kinase that is known to phosphorylate paxillin at Tyr31 and Tyr118, while ERK and JNK are serine/threonine kinases that phosphorylate residues Ser126 and Ser178, respectively (Huang et al. 2003). In the phosphorylation assays with semisynthetic **4a**, residue Tyr31, a site introduced by ligation, and residue Tyr118 were phosphorylated exclusively by Src; residue Ser126 was phosphorylated only by ERK; and residue Ser178 was phosphorylated only by JNK. The general anti-paxillin antibody recognized protein in all reactions. Importantly, detection of Src-treated **4a** by the pTyr31 phosphorylation-specific antibody validated both the successful ligation to install the Tyr31 site and the recognition of that site by an upstream kinase. As a further control, a full-length paxillin construct (GST-paxillin[1–557]-FLAG) was expressed, purified, and subjected to the kinase treatment, and the expressed paxillin responded identically to the semisynthetic version (data not shown). The binding and phosphorylation experiments confirm that the native interactions of the semisynthetic reconstituted control **4a**, including those of the phosphorylation site of interest, Tyr31, were not compromised by the expression and ligation procedures.

Uncaging of cpY31Pax (4c)

The extent of cpY31Pax (**4c**) uncaging was quantified following irradiation with long-wavelength UV light centered at 365 nm. Importantly for cellular applications, the photo-byproduct of NPE-caged species, *o*-nitrosoacetophenone, has previously been shown to be cellularly inert (Nguyen et al. 2004; Humphrey et al. 2005). This byproduct is less reactive than the analogous *o*-nitrosobenzaldehyde produced by irradiation of widely used *ortho*-nitrobenzyl-derived caging groups (Kaplan et al.

1978). For uncaging, **4c** was irradiated for 90 sec on a standard DNA transilluminator. The amount of phosphorylated paxillin (pY31Pax) present at t = 0 and t = 90 sec post-photolysis was quantified by chemiluminescent detection of the phosphorylated protein using a paxillin [pY31] phosphorylation-specific antibody. The total amount of protein loaded per blot was detected using a general anti-paxillin antibody. In addition, semisynthetic pY31 (**4b**) was used as a photochemically inert internal standard in the phosphorylation-specific and general paxillin Western blots (Fig. 4). The relative intensity of each sample was determined as a fraction of total pixel intensity compared to the internal pY31Pax (**4b**) standard. The ratio of uncaged phosphopaxillin to the total protein amount indicated 83%–91% conversion of **4c** to phosphoTyr31-paxillin after irradiation. A minimal amount of uncaged phosphoprotein, typically <6% of the total protein, was detected in the absence of intentional uncaging.

Herein, we have described the semisynthesis of paxillin analogs using an NCL strategy with N-terminal peptide thioesters, allowing access to multimilligram quantities of cpY31Pax (**4c**), pY31Pax (**4b**), and Y31Pax (**4a**). Binding and phosphorylation studies with **4a**, including FAK, GIT, and PTP-PEST binding and phosphorylation by Src, ERK, and JNK, confirmed that the semisynthetic paxillin derivatives function comparably in vitro to native paxillin. Uncaging experiments with **4c** verify the applicability of the probe for investigating downstream events of paxillin Tyr31 phosphorylation. The prevalence of reports of semisynthetic proteins modified on the C terminus compared to those modified at the N terminus has been attributed to the challenges of thioester generation following SPPS (Schwarzer and Cole 2005). The method used here is compatible with caged phosphoamino acids and, combined with the use of an Fmoc-protected caged phosphoTyr amino acid, requires only a single reaction (thioesterification) beyond standard SPPS protocols. This design allows the incorporation of a variety of unnatural amino acid mutations at the N terminus of paxillin.

To date, caged phosphorylated amino acids have been systematically incorporated into increasingly complex targets, including peptides and a protein domain. Herein, we have described the culmination of this progress with the assembly of a full-length, eukaryotic protein target. Ongoing cellular experiments using microinjected cpY31-Pax (**4c**) should yield important information on the effect of Tyr31 phosphorylation on cellular migration.

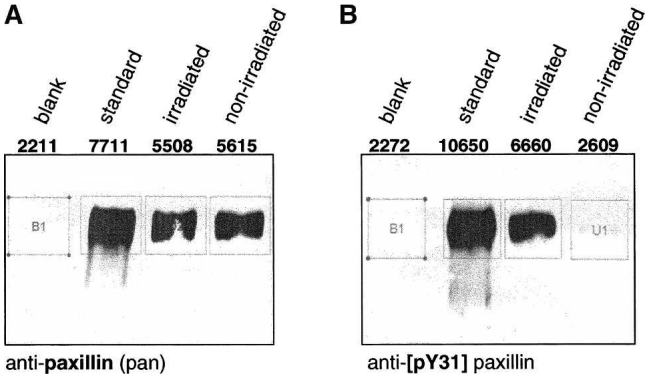

Figure 4. Western blot analysis of cpY31Pax (**4c**) irradiation to reveal pY31Pax. Following irradiation of **4c** with light centered at 365 nm, the protein was analyzed by Western blots probed with a general anti-paxillin(pan) antibody (*A*) and a phosphospecific anti-paxillin[pY31] antibody (*B*). Pixel counts for the intensity of each sample are shown in bold *below* the lane markers. Lanes: (B1) blank, (U3) semisynthetic pY31Pax (**4b**) as a standard for pixel counts, (U2) irradiated **4c**, (U1) nonirradiated **4c**. See Materials and Methods and Table 1 for a sample calculation of uncaging using this blot.

Materials and methods

Additional methods are available in Supplemental Methods online.

Thioester synthesis

Peptides were synthesized manually and on an automated (Advanced ChemTech 396) peptide synthesizer using standard (fluorenylmethoxy)carbonyl (Fmoc) solid-phase peptide synthesis (SPPS) protocols on preloaded Fmoc-Gly-Novasyn TGT resin (Novabiochem). Phosphotyrosine was introduced as Fmoc-Tyr(PO(OBzl)OH)-OH, and caged phosphotyrosine was introduced as Fmoc-phospho(1-nitrophenylethyl-2-cyanoethyl)-tyrosine. For acetylation of the amino terminus of each peptide, 120 μmol of peptide on resin was treated with acetic anhydride (113 μL, 1.2 mmol) and pyridine (97 μL, 1.2 mmol) in 10 mL of DMF for 30 min. The peptides were individually cleaved from the resin with side-chain protection intact by agitating with 0.5% TFA in DCM for 1.5 h. The resin was removed by filtration and rinsed with DCM, the solvent was mostly evaporated under a stream of nitrogen, and the peptide was triturated with cold hexanes. The hexanes were removed in vacuo, and the resulting white powder was dissolved in anhydrous THF (50 mL) and treated with HBTU (180 mg, 480 μmol), DIPEA (165 μL, 960 μmol), and benzylmercaptan (54 μL, 480 μmol) under nitrogen overnight. The THF was removed in vacuo and the peptide was deprotected with 95% (vol/vol) TFA, 2.5% (vol/vol) TIS, and 2.5% (vol/vol) water for 2 h. Peptides were triturated with cold diethylether and purified by reverse-phase HPLC on a Waters 600 instrument with a YMC C_{18} preparative column using an elution gradient of water/acetonitrile with 0.1% TFA. The identities of the peptides as free acids and of the final peptide thioester products were confirmed by electrospray ionization (ESI) mass spectrometry on a Perspective Biosystems Mariner Biospectrometry Workstation (turbo ion source).

Peptide characterization

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-Y-SYPTG-COOH, Reverse-phase HPLC (t_R = 25.4 min). Exact mass calculated for $C_{209}H_{307}N_{59}O_{68}$, 4731.2; found by MS(ESI), 947.8 $[MH_5]^{5+}$, 790.0 $[MH_6]^{6+}$.

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-Y-SYPTG-COSBn (**1a**), Reverse-phase HPLC (t_R = 25.7 min). Exact mass calculated for $C_{216}H_{313}N_{59}O_{67}S$, 4837.3; found by MS(ESI), 968.7 $[MH_5]^{5+}$.

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-pY-SYPTG-COOH, Reverse-phase HPLC (t_R = 25.4 min). Exact mass calculated for $C_{209}H_{308}N_{59}O_{71}P$, 4811.2; found by MS(ESI), 1204.6 $[MH_4]^{4+}$, 963.9 $[MH_5]^{5+}$.

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-pY-SYPTG-COSBn (**1b**), Reverse-phase HPLC (t_R = 25.6 min). Exact mass calculated for $C_{216}H_{314}N_{59}O_{70}PS$, 4917.2; found by MS(ESI), 1231.9 $[MH_4]^{4+}$, 985.7 $[MH_5]^{5+}$.

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-cpY-SYPTG-COOH, Reverse-phase HPLC (t_R = 25.4 min). Exact mass calculated for $C_{217}H_{315}N_{60}O_{73}P$, 4960.3; found by MS(ESI), 993.1 $[MH_5]^{5+}$.

Ac-HHHHHHDDLDALLADLESTTSHISKRPVFLSEETP-cpY-SYPTG-COSBn (**1c**), Reverse-phase HPLC (t_R = 25.7 min). Exact mass calculated for $C_{224}H_{321}N_{60}O_{72}PS$, 5066.3; found by MS(ESI), 1014.9 $[MH_5]^{5+}$, 845.9 $[MH_6]^{6+}$.

Plasmid construction for GST-paxillin(38–557)-FLAG

The gene fragment encoding residues 38–557 of paxillin (isoform α) was amplified from a paxillin plasmid (supplied by Martin Schwartz) with primers to insert 5′-EcoRI and 3′-NotI restriction sites. The primers also encoded an N-terminal TEV protease recognition site (ENLYFQC) and a C-terminal FLAG tag. For this amplification the following PCR primers were used: 5′-GCCGGAATTCGTGAAAACCTGTATTTTCAGTGC CACACATACCAGGAGATT-3′ and 5′-GCCCCCTTTTGCGG CCGCCTACTTATCGTCATCGTCTTTGTAGTCGCAGAAGA GCTTGAGGAA-3′. The PCR-amplified fragments were digested with NotI and EcoRI and ligated to NotI/EcoRI-digested and CIP-treated pGEX-4T-2 (GE Health Sciences). The ligation mixture was transformed into DH5α cells and grown on carbenicillin-resistant plates. Plasmid DNA was isolated from selected colonies and confirmed by sequencing.

GST-paxillin(38–557)-FLAG expression and purification

The paxillin plasmid was transformed into BL21-CodonPlus-RP competent cells (Stratagene) and grown with fermentation at 37°C to midlog phase in 10 L of LB media with carbenicillin and chloramphenicol. The culture was cooled to 16°C, and the cells were induced with 0.1 mM IPTG and fermented for 16 h. Cells were harvested by centrifugation and frozen at −80°C. For cell lysis, pellets were thawed and resuspended in 350 mL of lysis buffer (PBS, 1 mM $ZnCl_2$, 1 mg/mL lysozyme, 1 mM DTT, and Calbiochem protease cocktail III [100 μM AEBSF, 80 nM aprotinin, 5 μM bestatin, 1.5 μM E-64, 2 μM leupeptin, 1 μM pepstatin A]) and incubated for 20 min at 4°C. The cells were lysed with 1% NP-40 Alternative, then sonicated and subjected to centrifugation at 13, 000 rpm for 30 min, and at 35, 000 rpm for 30 min. The soluble fraction was purified using 8 mL of Glutathione Sepharose 4 Fast Flow resin following the manufacturer's protocol. Protein was dialyzed into TBS and then purified via the carboxy-terminal tag with 3 mL of anti-FLAG M2 affinity resin (Sigma). Typical yields for the doubly purified protein were 4–6 mg per 10 L fermentation, as quantified using a Biorad protein assay. The purified protein was stored at 4°C and used for all in vitro and cellular studies within 2 wk of lysis and purification.

TEV protease cleavage

The purified protein **2** was diluted to 0.5 or 1 mg/mL into a TEV cleavage buffer with a final concentration of 50 mM Tris pH 8.0, 500 μM EDTA, and 5 mM BME. TEV protease (US Biological) was added (35 μL of protease per mg of target protein), and the resulting solution was incubated at 28°C for 3 h. The protein was dialyzed into TBS (to remove glycine present from the FLAG-affinity elution) and incubated with Ni/NTA resin and glutathione sepharose beads to remove the hexahistidine-tagged TEV protease and the cleaved GST tag. The protein solution was concentrated using 50-kDa MWCO centrifugal filters (Millipore) and used immediately in NCL.

Ligations

In general, reactions were carried out with 50 μM protein, 0.8 mM peptide, and 100 mM MESNA in TBS at pH 8.0. Accordingly, to a solution of Cys-Pax(38–557)-FLAG (**3**) (600 μg, 10.7 nmol) in TBS (150 μL) was added Ac-HHHHHH-DDLDAL LADLESTTSHISKRPVFLSEETP-X-SYPTG-COSBn (lyophilized, then dissolved into 20 μL of water for transfer; 800 μg, 169 nmol for X = Tyr [**1a**], 163 nmol for X = pTyr [**1b**], and 158 nmol for X = cpTyr [**1c**]), 10 μL of 2 M MESNA, and 20 μL of 500 mM Tris pH 8.0. The reaction was incubated for 16 h at 25°C, and then dialyzed into PBS using a 50-kDa MWCO dialysis membrane to remove excess (4.8–5.1 kDa) peptide. Protein was either used directly for assays without a final purification or purified using a Ni/NTA spin column to isolate ligation product via the ligation-introduced N-terminal hexahistidine tag. The protein was analyzed by 10% SDS-PAGE gels and visualized with Coomassie blue dye, and by Western blot with a mouse anti-hexahistidine primary antibody. For ligations using **1b**, a mouse anti-pY31 antibody was also used for visualization.

Table 1. *Sample calculation of uncaging for cpY31Pax using data from Figure 4*

$$\%\ \text{uncaged} = \frac{\text{normalized intensity of total pY31 paxillin detected}}{\text{normalized intensity of total paxillin detected}}$$

% of pY31Pax uncaged at t = 0 sec = 0.040/0.619 = 0.06 = 6%

% of pY31Pax uncaged at t = 90 sec = 0.524/0.599 = 0.87 = 87%

Data table for the anti-paxillin (pan) probed Western blot

	Pixel count	Pixel count—blank	Normalized intensity
Blank (B1)	2211	—	—
pY31Pax (U3)	7711	5500	1
cpY31Pax, t = 90 (U2)	5508	3297	0.599
cpY31Pax, t = 0 (U1)	5615	3404	0.619

Data table for the anti-[pY31]paxillin (phosphospecific)-probed Western blot

	Pixel count	Pixel count—blank	Normalized intensity
Blank (B1)	2272	—	—
pY31Pax (U3)	10,650	8378	1
cpY31Pax, t = 90 (U2)	6660	4388	0.524
cpY31Pax, t = 0 (U1)	2609	337	0.040

Sample uncaging calculation for cpY31Pax

For uncaging, a 1.2 mg/mL solution of cpY31Pax (**4c**) in TBS with 2.5 mM DTT was irradiated for 90 sec in a glass cell (pathlength 1 mm) with light centered at 365 nm with an intensity of 7330 $\mu W/cm^2$ on a DNA transilluminator. Semisynthetic pY31Pax (**4b**) and equal amounts of caged and uncaged protein were run on 10% SDS gels and transferred to nitrocellulose. Western blots were developed with mouse (monoclonal) anti-human paxillin and phosphospecific rabbit (polyclonal) anti-paxillin[pY31] primary antibodies and visualized by chemiluminescence. Pixel counting was used to calculate the relative intensity, compared to a photochemically inert standard (**4b**), of phosphoprotein at $t = 0$ and $t = 90$ sec after uncaging of cpY31Pax (**4c**). A sample calculation is shown in Table 1 with data from the Western blot shown in Figure 4.

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