Photochemically-Activated Probes of Protein-Protein Interactions

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Supporting Information

EXPERIMENTAL PROCEDURES

Materials and chemicals were obtained from Fisher and Aldrich, unless otherwise indicated. 1-hydroxybenzotriazole (HOBt), benzotriazole-1-yloxytrispyrrolidinophosphonium hexa-fluorophosphate (PyBop), *N*,*N*,*N*_,*N*_-tetramethyl-*O*-(6-chloro-1*H*-benzotriazol-1-yl)uronium hexafluorophosphate (HCTU), 6-chloro-1-hydroxybenzotriazole dehydrate, (HOBt-Cl) *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5 _ *b*]pyridine-1-yl-methylene]-*N*-methylmethanaminiumhexafluorophosphate (HATU), 1-hydroxy-7-azabenzotriazole (HOAt), protected amino acids, and CLEAR Rink amide resin (100-200 mesh, 0.43 mmol/gram) were obtained from Advanced ChemTech, Bachem, Novabiochem, or Peptides International. $Pd(PPh_3)_4$ was obtained from Strem Chemicals. Dapoxyl sulfonyl chloride was obtained from Invitrogen. P-81 cellulose UNIFILTER plates were obtained from Whatman. GST-Lck-SH2 and PKA catalytic subunit plasmids were gifts from Dr. Qunzhao Wang and Dr. Hsien-Ming Lee, respectively. Fluorescence assays were performed using a Photon Technology QM-1 spectrofluorimeter, and irradiation experiments utilized an Oriel Mercury Arc Lamp (Model 69907) equipped with a 360 nm colored glass filter (300 - 400 nm band pass) and an IR filter.

Peptides Synthesis

All peptides were manually synthesized using a standard Fmoc solid-phase peptide synthesis protocol. The side chains of Glu and Ser were protected with *O*-*t*-Bu. The side chain of Arg was protected with the N^{ω}-(2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Pbf) group. The side chain group of phosphotyrosine was protected with *O*-benzyl. The side chain amine of (L)-2,3-diaminobutyric acid (Dab) residue was protected with the allyloxycarbonyl (Alloc) group.

Ac-pTyr-*N*(DMNB)Gly-Glu-Ile-Dab(Ac)-Ala-NH_2 (**4**) $C_{42}H_{60}N_9O_{18}P$, mass calcd *m/z* 1009.38, obsd 1009.66 (M+); Ac-N^{α}(DMNB)pTyr-Gly-Glu-Ile-Dab(Ac)-Ala-NH_2 (**5**) $C_{42}H_{60}N_9O_{18}P$, mass calcd *m/z*: 1009.38, obsd: 1007.62 (M-2); Ac-pTyr-Gly-Glu-Ile-Dab(Ac)-Ala-NH_2 (**6**) $C_{33}H_{51}N_8O_{14}P$, mass calcd *m/z* 814.33, obsd 812.05 (M-2); Ac-Gly-Gly-Gly-Phe-coumarin (**7**) $C_{29}H_{32}N_6O_8$, mass calcd *m/z*: 592.23, obsd: 593.86 (M+1); Ac-Gly-Gly-Gly-*N*(DMNB)Phe-coumarin (**8**) $C_{38}H_{41}N_7O_{12}$, mass calcd *m/z* 787.28, obsd 788.36 (M+1); H_2N-Leu-Arg-Arg-Ala-Ser-Leu-Gly-NH_2 (**9**) previously described (Mendelow, M.; Prorok, M.; Salerno, A.; Lawrence, D. S. *J. Biol. Chem.*, **1993**, *268*, 12289-96); H_2N-Leu-Arg-Arg-Ala-*N*(DMNB)Ser-Leu-Gly-NH_2 (**10**) $C_{41}H_{71}N_{15}O_{12}$, mass calcd *m/z* 966.10, obsd 968.40 (M+2); Ac-pTyr-Gly-Glu-Ile-Dab(Dapoxyl)-Ala-NH_2 (**11**) $C_{48}H_{63}N_{10}O_{16}P$, mass calcd *m/z* 1098.39, obsd 1097.03 (M-1).

Each amino acid was attached via a standard addition/deprotection stepwise protocol [steps (a) and (b)]. The reductive alkylation procedure (c) and coupling of the subsequent Fmoc-residue (d) were performed under the specified conditions, as was the coupling of the amino acid immediately following Ile in peptides **4** – **6** (e). Side chain deprotection of specific residues for subsequent modification (to furnish peptides **4** – **6**, **11**) is described in (f). Peptides were cleaved from the solid support as outlined in (g).

(a) Addition of standard Fmoc-amino acids to peptide chain: three equivalents of the Fmoc-protected amino acid, PyBOP, HOBt hydrate, and six equivalents of N-methylmorpholine (NMM) in DMF (3 hr).

(b) Deprotection of Fmoc group on the growing peptide chain: (i) 1 x 15 mL of 30% piperidine in DMF (30 min) (ii) 3 x 15 mL of DMF, (iii) 3 x15 mL of isopropyl alcohol, (iv) 3 x 15 mL of CH_2Cl_2.

(c) Reductive alkylation procedure for the incorporation of the DMNB caging group onto the peptide chain: the free primary amine peptide-resin (500 mg) was washed DMF/MeOH/AcOH (9:9:2) drained and then mixed with 3 equiv of 4,5-dimethoxy-6-nitrobenzaldehyde (DMNB) in 10 mL of DMF/MeOH (1:1) for 40 min. The solvent was then removed and the imine-forming reaction repeated. Following solvent removal, the resin was washed with 10 mL of DMF twice, and then 5 equiv of $NaBH_3CN$ in DMF/MeOH/AcOH (9:9:2) was added to the resin and mixed at room temperature for 20 min. The solvent was removed and the resin successively washed with DMF, DMF/H_2O, H_2O, MeOH/CH_2Cl_2, CH_2Cl_2, and DMF. The reaction was monitored by HPLC and ESI-MS analyses of cleaved product from a few mg of resin with 97% aqueous TFA. The reaction and the subsequent peptide synthesis were performed in reaction vessels that were wrapped in aluminum foil.

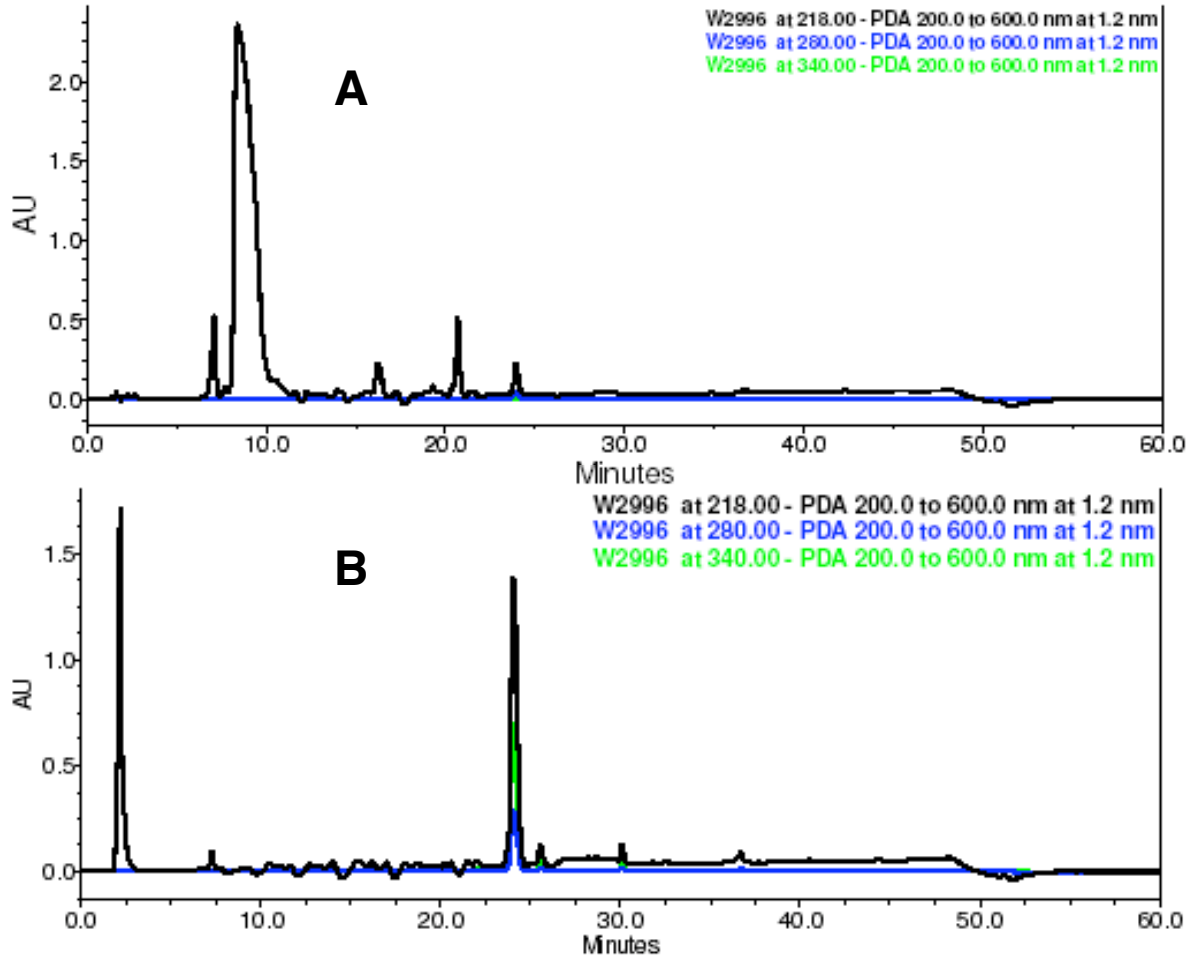


Figure S-1. Monitoring at 218 (black), 280 (blue) and 340 (green) nm of the reductive alkylation reaction by cleaving sample resins with 97% TFA. HPLC chromatogram of cleaved products before (**A**) and after (**B**) reductive alkylation of the Ser-Leu-Gly-resin in 20 min [0 - 50% CH_3CN over 45 min on an Apollo C18 (4.6 mm x 250 mm) analytical column].

(d) Amino acid coupling to the resin *N*-DMNB peptide: Peptides **5** and **8**: A solution of 6 equiv of the Fmoc-protected amino acid, HATU, and 18 equiv of DIPEA (diisopropylethylamine) in DMF was preactivated for 1 min. The solution was added to the resin-bound *N*-DMNB peptide and shaken for 2 hr. Peptide **10**: The acid chloride of Fmoc-Ala was prepared using a standard protocol (Carpino, L.A.; Cohen, B.J.; Stephens Jr., K.E.; Sadat-Aalaee, Y.; Tien, J.-H.; Langridge, D.C. *J. Org. Chem.*, **1986**, 51, 3732-34). Briefly, the Fmoc-amino acid was dried over phosphorus pentoxide in a desiccator overnight. A 250 mL round bottom flask was charged with 3.2 mmol of

Fmoc-Ala. 2.5 mL of $SOCl_2$ (ca. 32 mmol) was added via a cannula along with 23 μL of anhydrous DMF. The clear mixture was stirred for 1 h at room temperature. Excess $SOCl_2$ was evaporated. The acid chloride of Fmoc-Ala was crystallized by addition of 5 mL cold CH_2Cl_2 followed by 50 mL hexane. The crystals were filtered and dried over vacuum for 1 h. After washing thoroughly with more THF, 20 equiv. of freshly prepared Fmoc-Ala-Cl in THF (5 mL/1 g resin – previously swelled in THF for 1 h) was added and shaken for 30 min. 40 equiv. of DIPEA was subsequently added and the mixture allowed to react for an additional 2 h. The extent of racemization was determined by synthesizing the peptide containing the corresponding Fmoc-*D*-amino acid (i.e. Leu-Arg-Arg-*D*-Ala-N(DMNB)-Ser-Leu-Gly-amide) and comparing its retention times with that of the desired *L*-Ala derivative (Leu-Arg-Arg-*L*-Ala-N(DMNB)-Ser-Leu-Gly-amide). No significant racemization was detected using this coupling protocol.

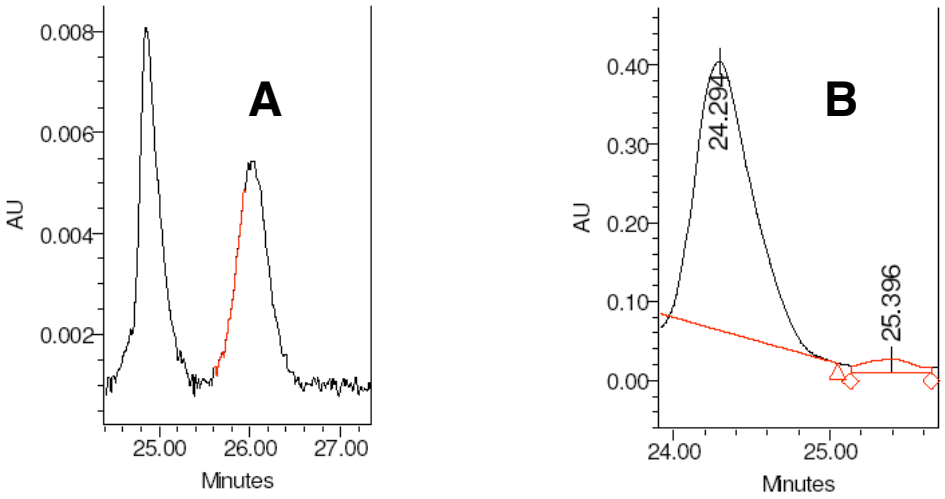
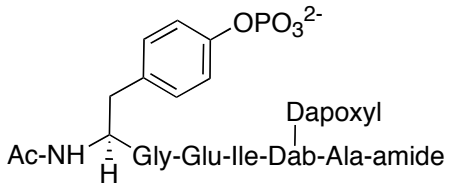


Figure S-2. An HPLC chromatogram of independently synthesized and co-injected Leu-Arg-Arg-*L*-Ala-N(DMNB)-Ser-Leu-Gly-amide (1st peak) and Leu-Arg-Arg-*D*-Ala-N(DMNB)-Ser-Leu-Gly-amide (2nd peak) (**A**). The peptides were resolved with an analytical column [Apollo C18 (4.6 mm x 250 mm)] using a slow gradient of 0 - 50% CH_3CN over 45 min. We observe approximately 4% racemization upon coupling of the acid chloride of Fmoc-L-Ala (**B**).

(e) Coupling of the amino acid immediately after Ile (**4**, **5**, **6)** was effected via initial exposure to the standard coupling conditions (i.e. with PyBop and HOBt), followed by a subsequent treatment with the amino acid to be coupled in the presence of HATU and HOAt.

(f) Following coupling of all the amino acids, the Dab side chain protected peptide-resin was treated with freshly prepared solution of $Pd(PPh_3)_4$ (1 equiv relative to resin substitution) in CH_2Cl_2/HOAc/NMM (48:1:1) to selectively remove the Alloc protecting group and expose the side chain Dab amine moiety. The resins were subsequently washed with 0.5% DIPEA followed by 0.5% sodium diethyldithiocarbamate in DMF. The peptide was then treated with acetic anhydride (4 equiv) and DIPEA (8 equiv) (**4**, **5**,

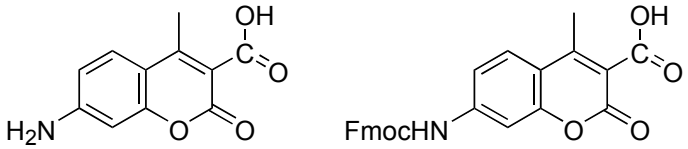
6) in dry CH_2Cl_2 and allowed to react overnight. Peptide **11** has been previously described (Wang, Q.; Lawrence, D. S. *J. Amer. Chem. Soc.*, **2005**, *127*, 7684-5).



11

(g) Peptides were cleaved from the resin (95% TFA, 2.5% triisopropylsilane, 2.5% H_2O), and purified by preparative reverse phase HPLC (Waters Atlantis dC18 19 mm X 100 mm) using a binary solvent system (solvent A: 0.1% TFA/H_2O; solvent B: 0.1% TFA/CH_3CN) with a ratio of (solvent A):(solvent B) that varied from 97:3 (0 min) to 75:25 (5 min) and then changed in a linear fashion to 65:35 (75 min).

Preparation of the Fmoc-coumarin derivative 13.



12 **13**

The free amine **12** was protected as the Fmoc derivative using a standard protocol (Backes, B. J.; Harris, J. L.; Leonetti, F.; Craik, C. S.; Ellman, J. A. *Nat. Biotechnol.* **2000** *18*, 187-93). ^{1}H NMR (DMSO): d 10.22 (s, 1H), 7.91-7.93 (d, *J* = 7.2 Hz, 2H), 7.73-7.78 (m, 3H), 7.55 (s, 1H), 7.34-7.46 (m, 5H), 4.55-4.57 (d, *J* = 6.6 Hz, 2H), 4.32-4.36 (t, *J* = 6.6 Hz, 1H), 3.58 (s, 2H), 2.36 (s, 3H). Derivative **13** was directly coupled to the Rink resin as described above in (a).

***K*$_d$ determinations of SH2 domain peptides**

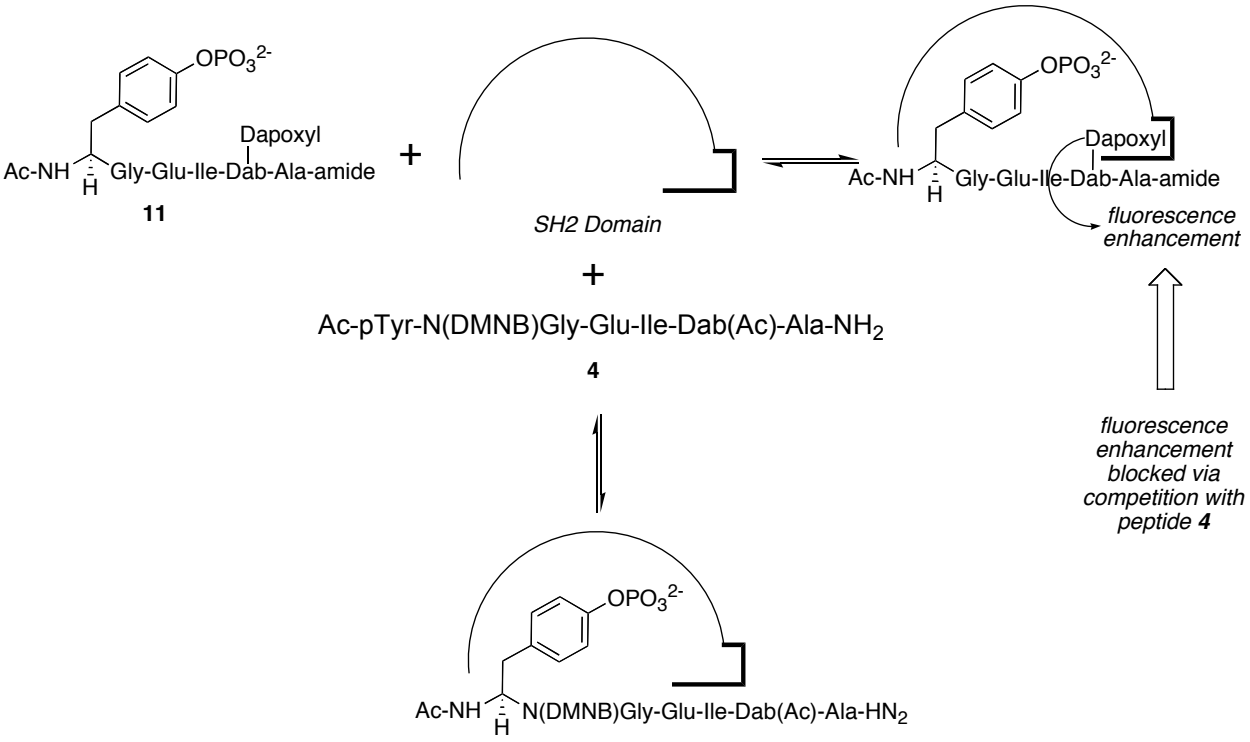
Experiments were conducted with 3 μM of peptide **11** sensor, 20 μM DTT, varied concentrations of GST-Lck-SH2 (in 10% glycerol) in 33.3 mM Tris buffer (pH 7.5) in an assay volume of 150 μL. The fluorescence of the solution was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC at λ_{ex} = 395 nm and λ_{em} = 535 nm. The fluorescence of the peptide solution was measured with variable concentration of GST-Lck-SH2 (0.1 μM, 0.2 μM, 0.4 μM, 0.8 μM, 1.6 μM, 3.2 μM, 6.4 μM, 12.8 μM, 25.6 μM). Control assays in the absence of GST-Lck-SH2 were also performed at the same concentrations. The K_d (1.5 ± 0.3) for the sensor peptide **11** was determined using the following equations.

$$K_d = \frac{([S_T] - [SP])([P_T] - [SP]}{[SP]}$$

where

$$[SP] = \frac{(F_x - F_o)}{(F_{max} - F_o)}[S_T]$$

and $[S_t]$ = total [**11**], F_x = fluorescence at specific [Lck-SH2], F_{max} = maximum fluorescence at excess [Lck-SH2], F_0 = starting fluorescence where [Lck-SH2] = 0, $[P_t]$ = total [Lck-SH2], [SP] = [**11**/Lck-SH2].



Scheme S-1. Competition assay to assess the K_d values of peptides **4** – **6**.

Subsequent K_d determinations for caged SH2 domain peptides **4** and **5**, and non-caged derivative **6** were performed via competition with peptide **11** (3 μM) in 20 μM DTT, 5 μM GST-Lck-SH2 (in 10% glycerol), and 33.3 mM of Tris buffer (pH 7.5) in a total assay volume of 150 μL. The fluorescence of the solution was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC at λ_{ex} = 395 nm and λ_{em} = 535 nm. The fluorescence of the peptide **11** solution was measured in the presence of variable concentrations of peptides **4**, **5**, or **6**. Control assays in the absence of GST-Lck-SH2 were also performed at the same concentrations. The following K_{dx} values were obtained: peptide **4** (127 ± 6 μM), peptide **5** (43 ± 10 μM), and peptide **6** (2.6 ± 0.2).

$$K_{dx} = \frac{[P]([X_T] - [XP])}{[XP]}$$

where

$$[P] = \frac{K_d[SP]}{[S_T] - [SP]}$$

$$[XP] = [P_T] - [P] - [SP]$$

$$[SP] = \frac{(F_x - F_o)}{(F_{max} - F_o)}[S_T]$$

and $[S_t]$ = total [**11**], F_x = fluorescence at a given concentration of peptide **X** (i.e. **4**, **5**, or **6**), F_{max} = fluorescence in the absence of peptide **X**, F_0 = fluorescence in the absence of Lck-SH2 domain, K_d = binding constant of **11**/Lck-SH2 domain, $[P_t]$ = total [Lck-SH2], [P] = uncomplexed [Lck-SH2], $[X_t]$ = total [**4**, **5**, or **6**], [PX] = [**4**, **5**, or **6**/Lck-SH2 complex], [SP]= [**11**/Lck-SH2 complex], K_{dx} = dissociation constant of peptide **4**, **5**, or **6**/Lck-SH2 domain complex.

Photolysis of caged peptides

Peptides **4** and **5**: experiments were conducted using 3 μM peptide **11**, 20 μM DTT, 5 μM GST-Lck-SH2 (in 10% glycerol), the caged peptide **4** or **5** (32 μM) in 33.3 mM Tris buffer (pH 7.5) and a total assay volume 150 μL [quartz cuvette; lightpath (10 mm) width (10 mm)]. The fluorescence of the solution was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC at λ_{ex} = 395 nm and λ_{em} = 535 nm. Caged peptide (**4**) was irradiated using an Oriel Mercury Arc Lamp (Model 69907) equipped with a 360 nm colored glass filter (300 - 400 nm band pass) and an IR filter for various time periods (0 – 30 min). An aliquot from each irradiation time period was added to the assay solution. A decrease in fluorescence intensity is indicative of displacement of the SH2 domain sensor **11** from the SH2 domain by photouncaged peptide (i.e. formation of **6**). The formation of uncaged peptide **6** from caged peptides **4** and **5** was confirmed by analytical HPLC (comparison with the retention time of peptide **6** prepared by solid phase peptide synthesis) and by mass spectrometry. Peptide **8** (25 μM) was irradiated as described above (0, 5, 10, 15, and 20 min exposure times) in 50 mM Tris buffer (pH 8.0), and 100 mM NaCl, 5 mM $CaCl_2$, and 0.01% Tween-20. Irradiated material was subsequently exposed to α-chymotrypsin (*vide infra*). Peptide **10** (30 μM) was photolyzed (*vide supra*) for different time periods (5, 10, 15, 20, 25, 30 min) and then subjected to PKA (*vide infra*).

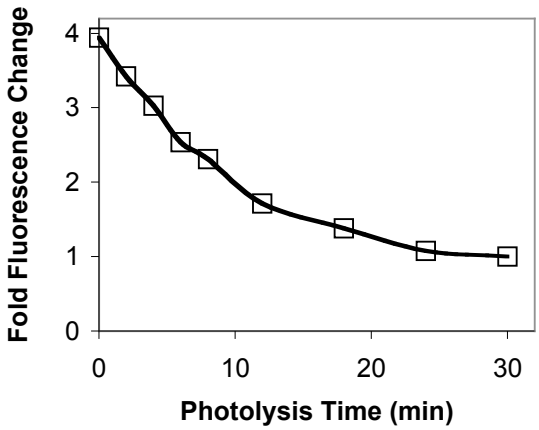


Fig. S-3. Fluorescence change as a function of photolysis time of caged peptide 4. At t = 0 min, the dapoxyl peptide **11** is completely bound to the Lck-SH2 domain and exhibits a 4-fold enhancement in fluorescence intensity relative to **11** in the absence of Lck-SH2 domain. Increasing irradiation times converts increasing quantities of **4** to active peptide **6** resulting ultimately in the complete displacement of **11** from the Lck SH2 domain.

Chymotrypsin Assay and Determination of K_m and V_{max} values with peptide 7

Assays were performed in triplicate using peptide **7** concentrations that varied about the K_m (0.39 μM, 0.78 μM, 1.56 μM, 3.12 μM, 6.25 μM, 12.5 μM, 25 μM) in 50 mM Tris buffer (pH 8.0), 100 mM NaCl, 5 mM $CaCl_2$, 10 nM α-chymotrypsin, and 0.01% Tween-20. The peptide **7** stock solution contained 5% DMSO and the assay solution contained less than 1% DMSO. The reaction was monitored on a Photon Technology QM-1 spectrofluorimeter at 30 ºC using a λ_{ex} = 380 nm and a λ_{em} = 460 nm.

Radioactive assay of the photolyzed PKA peptide 10

PKA assays were performed in triplicate. 20 μL of a photolyzed 30 μM solution of **10** was added to each well of 96 multi-well assay plate containing 20 μL assay buffer [100 mM MOPS, 150 mM KCl, 12.5 mM $MgCl_2$ and 150 μM cold ATP supplemented with 70 - 163 μCi/well [γ^{-33}P]ATP for radioactive detection]. 10 μL enzyme diluted buffer containing 100 mM MOPS (pH 7.1), 0.125 mg/mL bovine serum albumin, and 8 nM PKA catalytic subunit were added to initiate the reaction. Total reaction volume was 50 μL. After a 12 min incubation time at 30 ˚C, 100 μL of 6% phosphoric acid was added to each well to stop the reaction (total volume: 150 μL). Following an additional 5 min incubation period at ambient temperature, 75 μL from each reaction well was transferred into each well of a UNIFILTER (P81 cellulose phosphate paper) assay plate. Each well was washed four times with 0.1% phosphoric acid in water. Scintillation solution was added to each well and ^{33}P-incorporation measured by scintillation counting with a MicroBeta^{TM} TriLux & MicroBeta JET (Perkin Elmer). The formation of the photouncaged peptide was confirmed by ESI mass spectrometry. A time-based PKA assay with photolyzed peptide **10** was performed as described above except that the enzymatic reaction was stopped at different time points (2, 4, 6, 8, 10, 12, 14 min) by adding 6% phosphoric acid.