Supporting Information

Covalent Labeling of Fusion Proteins in Live Cells via an Engineered Receptor-Ligand Pair

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General Procedure and Materials

Cyclosporin A (CsA) was obtained from LC Laboratories (Woburn, MA). All other synthetic reagents were obtained from Aldrich Chemical (Milwaukee, WI) and used without further purification unless specified. Dichloromethane (CH_2Cl_2) was distilled from phosphorous pentoxide. Triethylamine (Et_3N) were distilled from calcium hydride. Dry dimethylformamide (DMF) was stored over AldraSORB. Deuteriochloroform (chloroform-*d*; $CDCl_3$) was stored over potassium carbonate before use. All moisture-sensitive reactions were performed in oven-dried glassware under a stream of nitrogen. Flash column chromatography (FCC) was performed on Silicycle silica gel 60 (230-400 mesh). DNA primers were obtained from Integrated DNA Technologies (Coralville, IA). Restriction enzymes were obtained from Promega (Madison, WI).

Semi-preparative purifications via reversed phase high performance liquid chromatography (RP-HPLC) were performed on a Beckman Coulter System Gold HPLC using a YMC-Pack ODS - A 100 x 10 mm column. Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 300 MHz using a Bruker AC+ 300 spectrometer. High-resolution electrospray ionization mass spectra (HRESI-MS) for small molecules were obtained on a Micromass LCT. LC/MS analysis of compounds was conducted on a Shimadzu 2010A LC-MS with a Supelco Discovery Biowide Pore C18-5 column (15 cm x 2.1 mm, 5 μm). Fluorescence gel images were obtained on a Typhoon Variable Mode Imager 9400 (GE Biosciences) using the green 532 nm laser for excitation and the 610BP30 emission filter (610 nm). An Olympus model IX81 motorized inverted fluorescence microscope equipped with a Chroma FITC (41001) and TRITC (41002c) filters was used for cell imaging.

***in Vitro* Labelling**

The P105C mutant of human cyclophilin A (P105C-CypA) was expressed and purified from *E. coli* as a glutathione-S-transferase (GST) fusion protein according to common procedures and as previously described[1]. A solution containing P105C-CypA at 5 μM in 50 mM phosphate buffered saline with 1 mM reduced glutathione and 0.05% Tween-20 was freshly prepared. CsA-EL-TMR was added to 10 μM from a 1 mM stock in ethanol. Portions (30 μl) were removed at indicated time points and added to 6x SDS-PAGE sample loading buffer[2] (6 μl) for 50 mM DTT and 0.2 % SDS final concentrations to stop the reactions. Band intensity was determined using the program ImageJ (NIH).

Labelling in Live Cells

Throughout experiments, cells were maintained in a 37 ^{o}C incubator with 5 % CO_2.

COS-7L cells were grown to ~75% confluence in cell culture treated 10 cm^2 well plates (GIBCO) in DMEM (Dulbecco's modified Eagle medium) supplemented with 10% fetal bovine serum and 5 mL/L of penicillin-streptomycin. Prior to transfection, cells were washed with phosphate buffered saline (PBS) and media was replaced with serum-free OPTI-MEM media (GIBCO). Indicated cells were then transiently transfected with P105C-CypA in the mammalian expression vector pcDNA4b (Invitrogen) using Lipofectamine 2000 (Invitrogen) according to the manufacture's instructions. After 10 hours of expression, media was exchanged back to supplemented DMEM and CsA-EL-TMR was added to 1 μM, if indicated. After 12 hours of incubation, cells were rinsed with PBS and immediately lysed with 250 μl 1x strength SDS sample loading buffer[2]. Lysates were passed repeatedly through a 1 inch, 25 gauge needle using a 1 mL syringe until viscosity was reduced sufficiently for SDS-PAGE. Band intensities were determined using the ImageJ program (NIH).

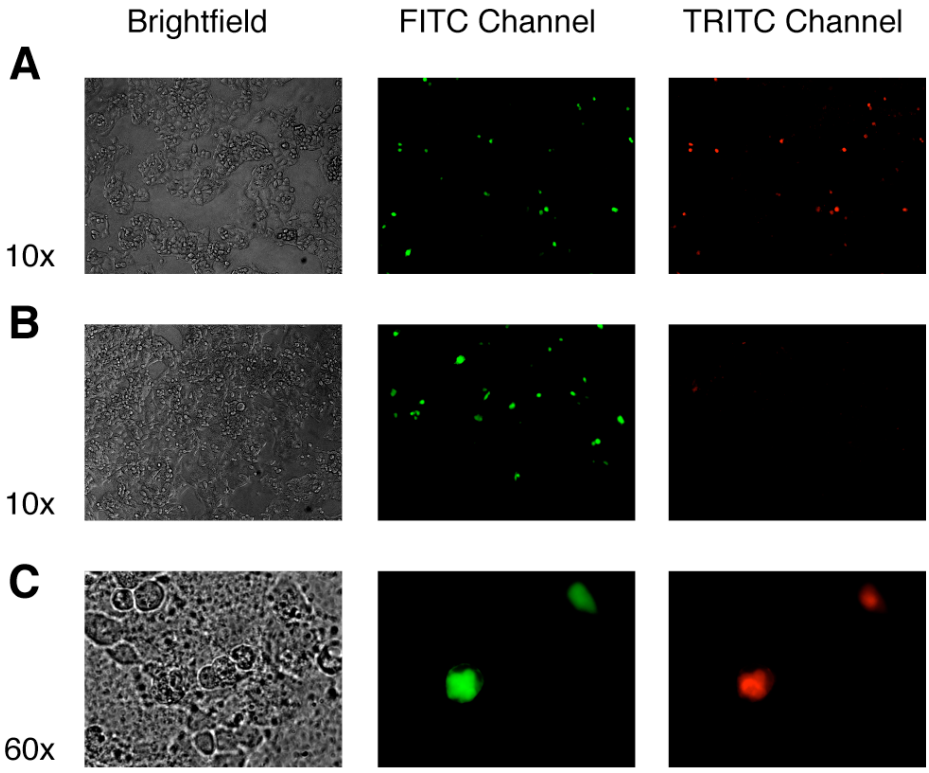
For microscopy experiments, Hep-G2 were grown in cell culture treated 2 cm^2 well plates (GIBCO) in supplemented DMEM medium as above. Cells were grown to ~75% confluence and media was exchanged to OPTI-MEM. Cells were then transfected as above but with a mammalian expression vector expressing a GFP-P105C-CypA fusion protein. After 24 hours of expression, media was exchanged back to supplemented DMEM. CsA-EL-TMR or CsA-Boc-TMR was added to 2.5 μM, where indicated. CsA was added to 2.5 μM. After 18 hours of incubation, cells were washed with PBS and fresh DMEM media was added. Over the course of a hour, media was exchanged an additional 2 times, lastly into OPTI-MEM. Cells were imaged directly in well plates and maintained at 37^{o} C and 5 % CO_2 throughout imaging using LiveCell system (Neue Biosciences) installed on the microscope stage. Images obtained were false colored appropriately as red or green using the ImageJ program (NIH).

Mammalian Expression Constructs

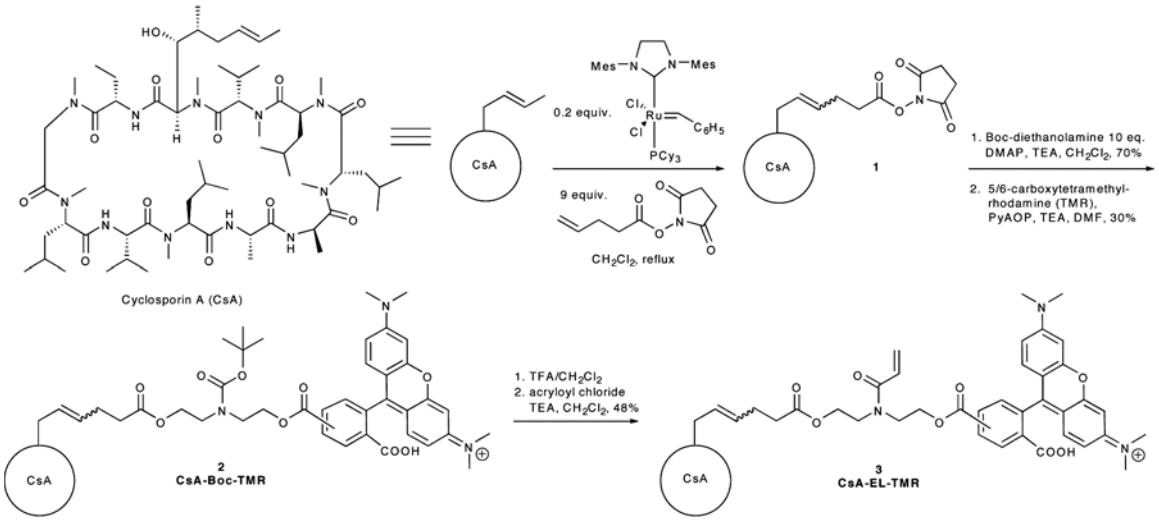
The P105C site-direct mutant was prepared by QuikChange (Stratagene) mutagenesis with previously described[1] primers from a pBJ5E plasmid[3] containing human CypA (K. Levitsky and P. J. Belshaw, unpublished: plasmids and sequences available upon request). P105C-CypA was cloned from the pBJ5E vector using the primers: 5'-ATCTGAATTCGTTATGGCCCTCGAGGTCAACCCCACC-3' and 5'-CTCGAAGTCGACGCGGCCGCCTGATCTAGATAAATA-3'. PCR product was digested with EcoRI and XbaI and inserted into pcDNA4B (Invitrogen).

For green fluorescent protein (GFP)-P105C-CypA fusion constructs, GFP was cloned from the pIRES-eGFP plasmid (Clontech) using the primers: 5'-ATCTGAATACGTTATGGTGAGCAAGCG-3' and 5'-TATTTACTCGAGCTTGTACAGCTCGTCCAT-3'. PCR product was digested with EcoRI and XhoI and inserted into the previously prepared pcDNA4B plasmid with P105C-CypA to produce a GFP with a C-terminal P105C-CypA tag.

Colour version of Figure 3



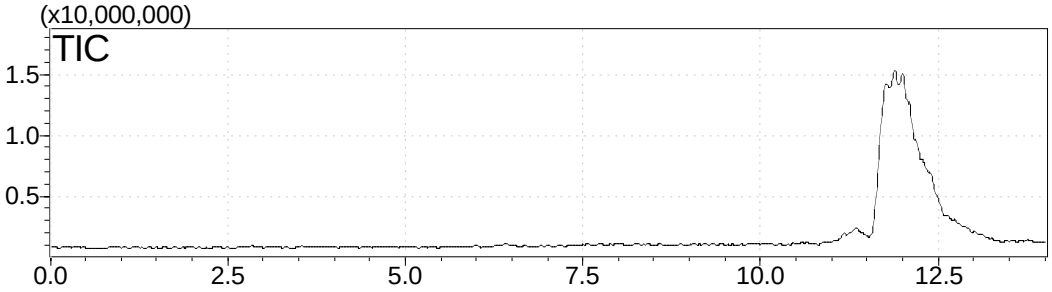
Synthesis of CsA Derivatives



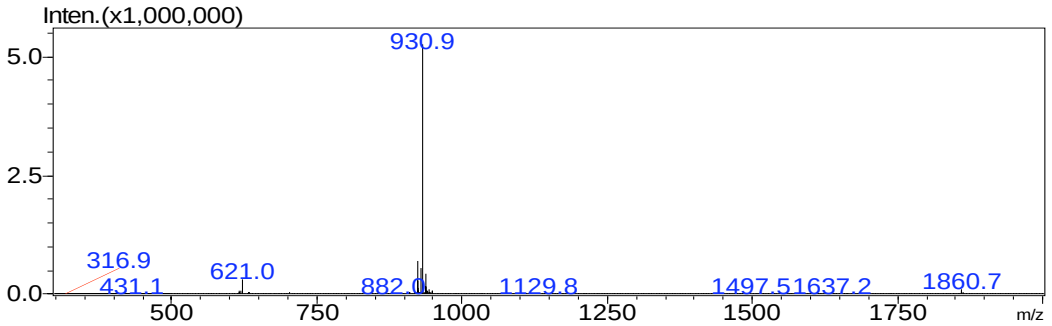
CsA Boc-Diethanolamine. CsA active ester derivative, **1**, was prepared as previously described. A solution of **1** (20 mg, 15 μmol) in dry CH_2Cl_2 (1.5 mL) was added to boc-diethanolamine (30 mg, 150 μmol), DMAP (0.5 mg, 2.9 μmol), and TEA (8.2 μl, 59 μmol). The reaction was allowed to proceed overnight under N_2. The mixture was purified silica gel chromatography with 60% EtOAc in Et_2O to yield the final free alcohol compound (14.9 mg, 70%). R_f = 0.19 (100 % EtOAc). ^{1}H NMR ($CDCl_3$, 300 MHz) δ 8.08 (d, *J* = 8.3 Hz, 1H), 7.71 (d, *J* = 6.7 Hz, 1H), 7.52 (d, *J* = 7.9 Hz, 1H), 7.18 (d, *J* = 7.2 Hz, 1H), 5.69 (dd, *J* = 11.3, 3.5 Hz, 1H), 5.38-5.50 (m, 2H), 5.26-5.35 (m, 2H), 4.94-5.13 (m, 4H), 4.77-4.90 (m, 1H), 4.61-4.75 (m, 2H), 4.45-4.56 (m, 1H), 4.18 (broad s, 2H), 3.71-3.80 (m, 3H), 3.49 (s, 3 H), 3.42-3.45 (m, 3H), 3.40 (s, 3 H), 3.25 (s, 3H), 3.15-3.23 (m, 1H), 3.12 (s, 3H), 3.10 (s, 3H), 2.7 (s, 3H), 2.68 (s, 3H), 2.25-2.47 (m, 4H), 1.94-2.18 (m, 6H), 1.70-1.88 (m, 4H), 1.55-1.69 (m, 6H), 1.46 (s, 9H), 1.43 (s, 2H), 1.34 (d, *J* = 7.2 Hz, 6H), 1.26 (d, *J* = 6.9 Hz, 6H), 0.91-1.08 (m, 25H), 0.75-0.90 (m, 20H), 0.68-0.74 (m, 4H); MS (HRESI-MS) calculated for [$C_{73}H_{130}N_{12}O_{17}$ + Na^+] 1469.9575 m/z, found 1469.9559 m/z.

CsA-Boc-TMR, 2. CsA-Boc-diethanolamine (10.1 mg, 7.0 μmol), 5(6)-carboxytetramethylrhodamine (TAMRA-mixed isomers) (9.0 mg, 21 μmol), 7-Azabenzotriazol-1-yloxy)tripyrrolidinophosphonium (PyAOP) (10.9 mg, 21 μmol), and triethylamine (5.0 μL, 35.6 μmol) were combined in dry DMF (~0.5 mL). The solution was reduced to a minimal volume in a 1 dram vial with N_2. An additional portion (5.0 μL) of TEA was added and the reaction was allowed to proceed for 2 days at RT. The mixture was purified by reverse phase HPLC with H_2O (as solvent A) and MeOH (as solvent B) using the following gradient: 40 % B for 1 min, 40-98% B over 19 min, 98% B for 10 min. Solvent was evaporated from the collected solution under reduced pressure and the remaining solution was lyophilized to yield the final compound, CsA-Boc-TMR (3.9 mg, 30 %). MS (HRESI-MS) calculated for [$C_{98}H_{151}N_{14}O_{21}^+$ + Na^+]$^{2+}$ 941.5538 m/z, found 941.5519 m/z. To further establish the purify of the compound, it was

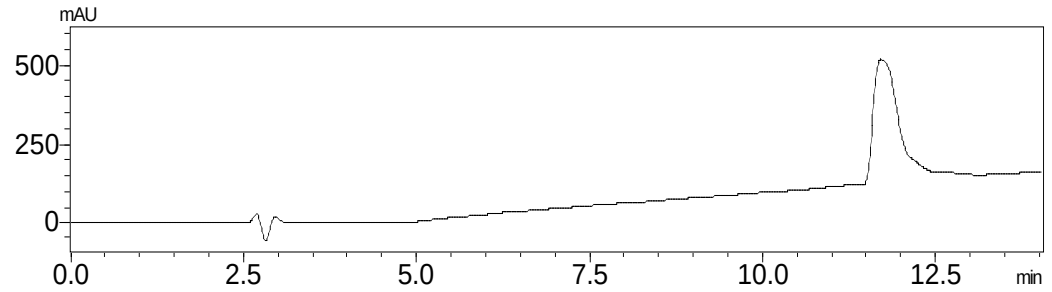
characterized by LC/MS. The sample was run with a heated column at 80 ^{o}C to minimize peak broadening. H_2O with 0.1 % formic acid (as solvent A) and ACN with 0.1 % formic acid (as solvent B) were used as the mobile phase with the following gradient: 30% B for 1 min., 30-95% B over 11 min., 95% B for 3 min., and 95-30% B over 1 min.



Total Ion Current (TIC)



MS spectrum from 11.5 to 12.6 min

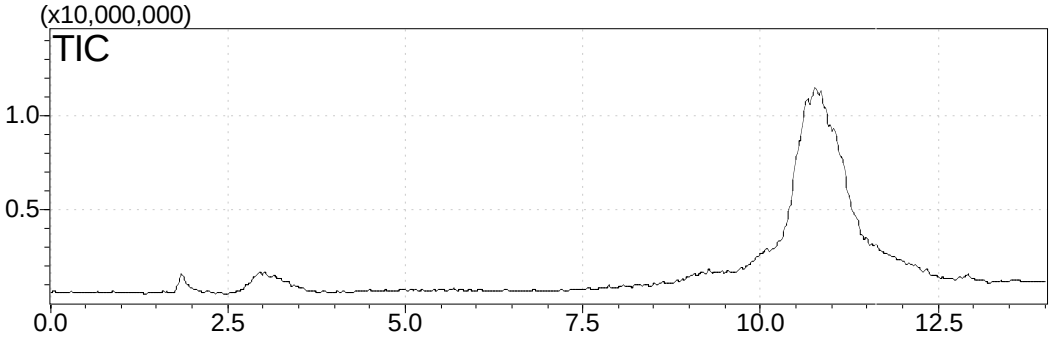


Abs. at 220 nm

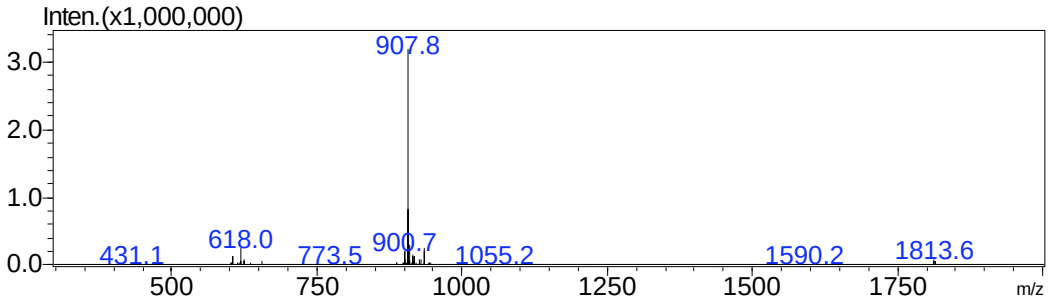
CsA-EL-TMR, 3. CsA-Boc-TMR, 2, (1.7 mg, 0.93 μmol) was dissolved in DCM (~500 μL) mixed with trifluoroacetic acid (500 μL). The mixture was stirred at RT for 3 hours and then solvent was removed under reduced pressure. Compound was resuspended in DCM and was directly evaporated again to remove residual TFA. Compound was placed under high vacuum overnight and carried on without further purification.

A solution of acryloyl chloride (1.3 μmol) was prepared in dry DCM (0.5 mL) and stirred in a 1 dram vial on ice. The deprotected CsA-Boc-TMR was dissolved in DCM and added to triethylamine (1 μL). This mixture was added slowly, dropwise to the stirring acryloyl chloride on ice. The reaction was allowed to react and come to RT

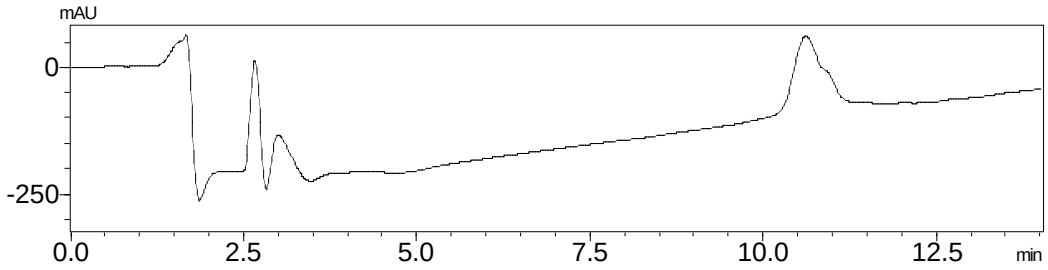
overnight. The resulting mixure was purified on reverse phase HPLC as above to yield the final CsA-EL-TMR compound (0.8 mg, 48 %). MS (HRESI-MS) calculated for $[C_{95}H_{145}N_{14}O_{19}^{+} + Na^{+}]^{2+}$ 918.5328 m/z, found 918.5346 m/z. To further establish purity, the compound was analyzed by LC/MS performed as with compound **2**.



Total ion current (TIC)



Mass spectrum from 10.5 to 11.4 min.



Abs at 220 nm

References:

1. K. Levitsky, C. J. Ciolli and P. J. Belshaw, *Org. Lett.*, 2003, **5**, 693.
2. *Short Protocols in Molecular Biology,* Edited by F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl, John Wiley & Sons, Inc. New York, 4th edn., 1999.
3. P. A. Clemons, B. G. Gladstone, A. Seth, E. D. Chao, M. A. Foley and S. L. Schreiber, *Chem. Biol*., 2002, **9**, 49.