Transglutaminase-Catalyzed Site-Specific Conjugation of Small-Molecule Probes to Proteins in Vitro and on the Surface of Living Cells

Supporting Information

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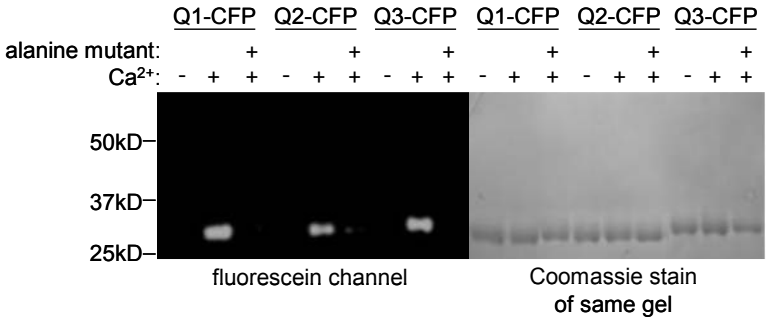

Figure S1. TGase-catalyzed labeling of Q-tagged CFP proteins in vitro with fluorescein cadaverine. Three Q-tagged proteins, Q1-CFP, Q2-CFP, and Q3-CFP, were incubated with gpTGase, $CaCl_2$, and fluorescein cadaverine for 30 min at 4 °C. The products were visualized on 12% SDS-PAGE by fluorescence and Coomassie stain. Fluorescein conjugation to Q-tag-CFP proteins is seen in the presence of Ca^{2+}, but not when Ca^{2+} is omitted. Protein mutants with glutamine to alanine substitutions in their Q-tags were also not labeled.

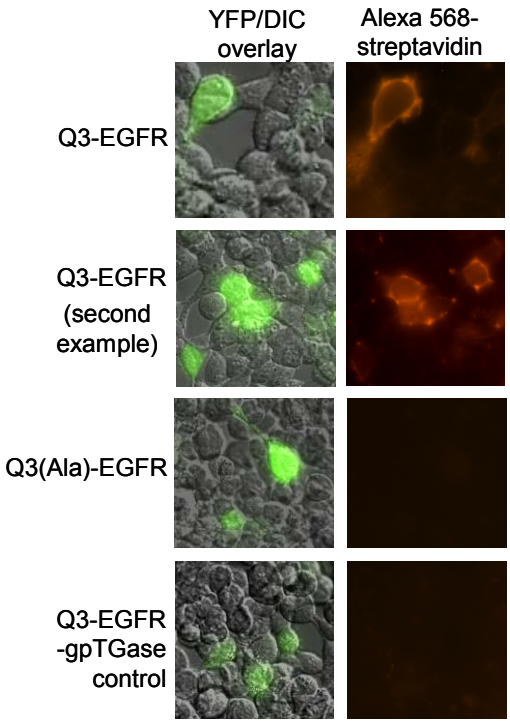

Figure S2. TGase-catalyzed labeling of Q3-EGFR with biotin cadaverine. HEK cells co-expressing Q3-EGFR (EGF receptor with an N-terminal extracellular Q3 tag), or its alanine mutant, and a cytoplasmic YFP transfection marker were labeled with biotin cadaverine by incubating with probe, gpTGase, and $CaCl_2$ for 30 min at 37 °C. Biotin incorporation was detected by staining with streptavidin conjugated to Alexa 568. The left column shows the YFP channel images superimposed on the DIC images. The right column shows the Alexa 568 fluorescence from streptavidin staining. Negative controls are shown with the alanine mutant Q3(Ala)-EGFR or with gpTGase enzyme left out.

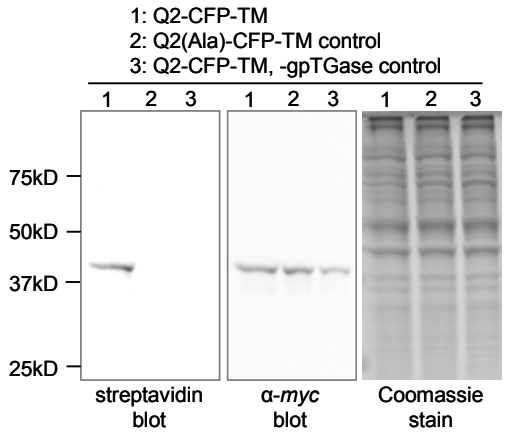

Figure S3. Biotin cadaverine labeling of cell surface Q2 and analysis by streptavidin blot. HeLa expressing Q2-CFP-TM were incubated with biotin cadaverine, gpTGase, and $CaCl_2$ for 25 min at 4 °C. Cells were then lysed, run on 12% SDS-PAGE, and blotted with streptavidin or anti-*myc* antibody (as a control for equal expression). Biotin conjugation to Q2-CFP-TM (MW 37 kD) is seen in lane 1 but not in lanes 2 and 3 where the alanine mutant was used, or where gpTGase was omitted. The bands ordinarily seen from endogenous biotinylated proteins[1] are not visible presumably because their concentrations are low compared to overexpressed Q2-CFP-TM.

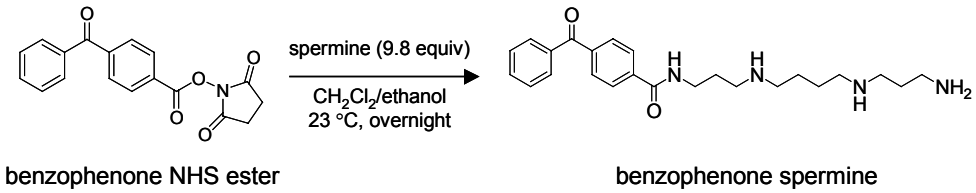

Figure S4. Synthesis of benzophenone spermine.

Experimental Protocols

Q-tag-CFP gene construction, expression, and protein purification. The following primers were used to amplify the CFP gene: 5′-ccggatccgcccgggccaaacccacaactaccattcgagtcctccggcggcatgg and 5′-ggggaattcttaccgcggcttgtacagctcgtc (Q-tag CFP.R). These introduced the Q1 tag (amino acid sequence PNPQLPF) in-frame onto the C-terminus of CFP. The PCR product was digested with *BamHI* and *EcoRI* enzymes and ligated into the pRSETB vector (Invitrogen). To introduce the Q2 tag (amino acid sequence PKPQQFM), the following primers were used: 5′-ccggatccgcccgggccaaaaccacaacaattcatggagtcctccggcggcatgg and Q-tag CFP.R. To introduce the Q3 tag (amino acid sequence GQQQLG), the following primers were used: 5′-ccggatccgcccgggggccaacaacaactaggcgagtcctccggcggcatgg and Q-tag CFP.R. For protein expression, the plasmids were introduced into the bacterial strain BL21(DE3) (Stratagene) by heat-shock transformation. The cells were grown in Luria Broth supplemented with ampicillin (100 μg/mL) at 37 ºC until OD_{600} 0.5. IPTG was then added to a final concentration of 0.4 mM to induce protein expression. The cells were grown for an additional 3 h at 30 ºC and then harvested by centrifugation. Cells were lysed by sonication at 4 ºC (six 30-second pulses at half-maximal power with 1 minute in between each pulse) in lysis buffer (50 mM Tris pH 7.8, 300 mM NaCl, 4 mM PMSF, and ¼ EDTA-free protease inhibitor cocktail tablet (Roche) per 10 mL of lysis buffer). The His_6-tagged protein was purified from the lysate using a Ni-NTA agarose (Qiagen) column following the manufacturer's instructions. Fractions containing the proteins were consolidated and transferred into TBS (140 mM NaCl, 3 mM KCl, 25 mM Tris pH 7.4) by two rounds of dialysis for storage in aliquots at -80 ºC. Typical yields were 0.5-1 mg of protein per 0.5 L culture. Alanine mutants were constructed by the same cloning procedure described above, using the following forward primers: 5′-ccggatccgcccgggccaaacccagccctaccattcgagtcctcc for Q1(Ala)-CFP, 5′-ccggatccgcccgggccaaaaccagccgccttcatggagtcctcc for Q2(Ala)-CFP, and 5′-ccggatccgcccgggggcgccgccgccctaggcgagtcctccg for Q3(Ala)-CFP.

Construction of the Q-tag-CFP-TM genes. The CFP gene was PCR-amplified using the following primers: 5′-ggcccgggccaaacccacaactaccattcgagtcctccggcggcatgg and Q-tag CFP.R for Q1-CFP-TM; 5′- ggcccgggccaaaaccacaacaattcatggagtcctccggcggcatgg and Q-tag CFP.R for Q2-CFP-TM; and 5′- ggcccgggggccaacaacaactaggcgagtcctccggcggcatgg and Q-tag CFP.R for Q3-CFP-TM. The PCR products were digested with *XmaI* and *SacII* and ligated into the pDisplay vector (Invitrogen). To construct the alanine mutant of Q1-CFP-TM, QuikChange (Stratagene) was performed using the primer 5′-ccaaacccagccctaccattcgag and its reverse complement. Q2(Ala)-CFP-TM was constructed via PCR of CFP with the primers 5′-cgggccaaaaccagcagcattcatggagtcc and Q-tag CFP.R, followed by digestion and ligation into pDisplay. Q3(Ala)-CFP-TM was constructed via PCR of CFP with the primers 5′- gatctcccgggggcgcagcagcactaggcgagtcctc and Q-tag CFP.R, followed by digestion and ligation into pDisplay.

Construction of the Q3-EGFR gene. PCR was performed with the AP-EGFR pcDNA3 plasmid[2] as template, the T7 primer, and a primer with sequence 5′-cggctagcgcccttgtc

atcatcgtctttgtaatcgccgccgcctagttgttgttggccagcccgactagc. The PCR product was digested with *XhoI* and *NheI* and ligated into similarly cut AP-EGFR pcDNA3. The alanine mutant was constructed by PCR amplification of the EGFR gene with the T7 primer and 5′-cggctagcgcccttgtcatcatcgtctttgtaatcgccgccgcctagtgctgctgcgccagcccgactagc, followed by digestion and ligation into similarly cut AP-EGFR pcDNA3.

Labeling of Q-tag-CFP in vitro. Reaction conditions were as follows: 6.3 μM Q-tag-CFP, 20 mM HEPES pH 8.2, 10 mM $MgCl_2$, 0.1 mM EGTA, 5 mM $CaCl_2$, 0.5 mM fluorescein cadaverine (Molecular Probes), and 25 ng/μL guinea pig liver transglutaminase (gpTGase, Sigma). To quantify labeling specificity, we used ImageQuant software (Amersham Biosciences) to determine the background-corrected fluorescence intensity for each band in the gel. The signal to background ratio was determined by dividing the intensity of the Q-tag CFP band by the intensity of its corresponding alanine mutant band. Analyses were performed on three independent labeling experiments. To estimate the extent of labeling, Q1-CFP was incubated with biotin cadaverine under conditions otherwise identical to those given above. Fully biotinylated CFP-AP (enzymatically modified by biotin ligase)[2] was used as a reference standard. Samples were run on 12% SDS-PAGE and then blotted with horse radish peroxidase (HRP)-conjugated streptavidin (1: 4000 dilution; Bio-Rad). A standard curve for CFP-AP was generated using ImageQuant software. Analyses were performed on three independent labeling experiments.

Labeling of cell surface Q-tag-CFP-TM with Alexa 568 cadaverine. HeLa cells maintained in 10% fetal bovine serum in DMEM (Invitrogen) at 37 ºC under 5% CO_2 were transfected with the Q-tag-CFP-TM plasmids using Lipofectamine 2000 (Invitrogen). After 12-24 h, the cells were cooled for 10 min in 4 ºC DPBS, then labeled for 25 min at 4 ºC with 0.4 mM Alexa 568 cadaverine (Molecular Probes), 12 mM $CaCl_2$, 1 mg/mL BSA, and 50 ng/μL gpTGase in DMEM. The cells were rinsed three times with 4 ºC DPBS, then imaged on a Zeiss Axiovert 200M inverted epifluorescence microscope with CFP (420DF20 excitation, 450DRLP dichroic, 475DF40 emission), Alexa 568 (560DF20 excitation, 585DRLP dichroic, 605DF30 emission), and DIC (775DF50 emission) filter sets. Images were acquired for 0.5-0.9 sec using OpenLab software (Improvision). To quantify labeling specificity, regions of interest (ROIs) were selected on 42 cells expressing either Q2-CFP-TM or Q2(Ala)-CFP-TM, using OpenLab software. ROIs were selected to overlap with cell membranes, and to exclude non-membrane space. For each ROI, both the Alexa 568 and CFP mean intensities were recorded and graphed against each other. Given a fixed CFP intensity, the signal to background ratio was calculated by dividing Alexa 568 intensity for a Q2-CFP-TM ROI by that for a Q2(Ala)-CFP-TM ROI.

Labeling of cell surface Q3-EGFR with biotin cadaverine. HEK293 cells maintained in 10% fetal bovine serum under 5% CO_2 were co-transfected with Q3-EGFR and YFP-pcDNA3 plasmids in a 10:1 ratio using Lipofectamine 2000. Cells were labeled with 0.5 mM biotin cadaverine, 12 mM $CaCl_2$, and 25 ng/μL gpTGase in DMEM at 37 ºC for 30 min. The biotin was then detected with Alexa 568-conjugated streptavidin (1:200

dilution, Molecular Probes) in DPBS for 22 min at room temperature. Cells were imaged as described above.

Synthesis of benzophenone spermine. To a stirred solution of spermine (1.26 g, 6 mmol, 9.8 equiv) in ethanol (5 mL), benzophenone *N*-hydroxysuccinimide (NHS) ester (prepared as reported,[3] 205 mg, 0.63 mmol, 1 equiv) in CH_2Cl_2 (3 ml) was added. The reaction was allowed to proceed overnight at room temperature. The mixture was then concentrated in vacuo and purified by silica chromatography using 1:1 methanol-CH_2Cl_2, then 2:2:0.5 methanol-CH_2Cl_2-NH_4OH, then 2:2:1.5 methanol-CH_2Cl_2-NH_4OH, to yield benzophenone spermine (13 mg, 5%). TLC: R_f = 0.22 (2:2:1.5 methanol-CH_2Cl_2-NH_4OH). ^{1}H-NMR (400 MHz, D_2O): δ 7.88 (s, 4H), 7.82 (d, 2H, *J* = 7.6), 7.74 (t, 1H, *J* = 6.8), 7.59 (t, 2H, *J* = 7.2), 3.43 (t, 2H, *J* = 6.4), 3.00 (m, 10H), 1.95 (m, 4H), 1.69 (m, 4H). ESI-MS m/z: (pos) 411.2 $[M+H]^+$.

p50-Q2 gene construction, expression, and protein purification. The p50 gene (murine, a gift from G. Ghosh, UCSD) was PCR-amplified (from amino acid 41 to 350) with the primers: 5′-`cccatatggattacaaagacgatgatgacaagggcgctagcggcccataccttc` (FLAG-p50.F) and 5′-`ggggatccctacatgaattgttgtggttttggttcagggtagtagagaaaggg`. The PCR product was digested with *NdeI* and *BamHI* and ligated into the pET-15b vector (Novagen). The alanine mutant (p50-Q2(Ala)) was constructed similarly, but using the primers FLAG-p50.F and 5′-`ggggatccctacatgaaggcggctggttttggttcagggtagtagaga aaggg`. For protein expression, the plasmids were introduced into the bacterial strain BL21(DE3) by heat-shock transformation. The cells were grown in Luria Broth supplemented with ampicillin (100 μg/mL) at 37 ºC until OD_{600} 0.3. IPTG was then added to a final concentration of 0.4 mM to induce protein expression. The cells were grown for an additional 15 h at room temperature and then harvested by centrifugation. Cells were lysed by sonication at 4 ºC (six 30-second pulses at half-maximal power with 1 minute in between each pulse) in lysis buffer (50 mM Tris pH 7.8, 300 mM NaCl, 4 mM PMSF, and ¼ EDTA-free protease inhibitor cocktail tablet per 10 mL of lysis buffer). The His_6-tagged protein was purified from the lysate using a Ni-NTA agarose column following the manufacturer's instructions. Fractions containing the recombinant proteins were consolidated and transferred into TBS by two rounds of dialysis for storage in aliquots at -80 ºC. Typical yields were 5-10 mg of protein per 0.5 L culture.

Photocrosslinking assay for p50-Q2. Proteins were labeled in vitro with benzophenone spermine as follows: 20 μM p50-Q2, 10 mM $CaCl_2$, 1 mM benzophenone spermine, 100 ng/μL gpTGase, and 50 mM DTT in PBS pH 7.4 for 1.5 h at 37 ºC. The proteins were then re-purified to remove excess benzophenone using Ni-NTA agarose, with a wash buffer consisting of 50 mM Tris pH 7.8, 300 mM NaCl, and 30 mM imidazole, and an elution buffer consisting of 50 mM Tris pH 7.8, 300 mM NaCl, and 100 mM imidazole. The proteins were re-concentrated with YM-10 centricon filters (Millipore). For cross-linking reactions, 1 μM benzophenone-labeled protein was irradiated for 7 min at 4 ºC (800 W lamp from Hanovia). To promote p50-Q2 dimerization, 0.18 μM duplex DNA (pre-formed by annealing κB#SeqB.F: 5′-`gtaggggcctccccggctcgagatcctatg` and κB#SeqB.R: 5′-`cataggatctcgagccggggaggcccctac`)[4] or 360 μM myotrophin[5] was added and incubated with the sample for 20 min at room temperature before UV

exposure. The samples were analyzed by 10% SDS-PAGE and blotting with anti-p50 antibody (1:1000 dilution, Biomeda, Forster City, CA) followed by HRP-conjugated anti-rabbit antibody (1:1000 dilution, Bio-Rad). The HRP signal was visualized with Supersignal West Pico (Pierce).

References

(1) Howarth, M.; Takao, K.; Hayashi, Y.; Ting, A. Y. *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 7583-7588.

(2) Chen, I.; Howarth, M.; Lin, W. Y.; Ting, A. Y. *Nat. Methods* **2005**, *2*, 99-104.

(3) Parker, J. M. R.; Hodges, R. S. *J. Protein Chem.* **1984**, *3*, 465-478.

(4) Pan, J.; McEver, R. P. *J. Biol. Chem.* **1995**, *270*, 23077-23083.

(5) Knuefermann, P.; Chen, P.; Misra, A.; Shi, S. P.; Abdellatif, M.; Sivasubramanian, N. *J. Biol. Chem.* **2002**, *277*, 23888-23897.