CHEMBIOCHEM

Supporting Information

© Copyright Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2006

© Copyright Wiley-VCH Verlag GmbH & Co. KGaA, 69451 Weinheim, 2006

CHEMBIOCHEM

Supporting Information

for

C-Terminal Fluorescence Labeling of Proteins for Interaction Studies on the Single Molecule Level

Christian F.W. Becker, Ralf Seidel, Michael Jahnz, Kirsten Bacia, Thomas Niederhausen, Kirill Alexandrov, Petra Schwille, Roger S. Goody, and Martin Engelhard

Experimental Section

9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) and Fmoc-His(Trt)-Wang resin were purchased from Novabiochem. Trifluoroacetic acid was from Riedel-de Häen. Fmoc-Lys-ivDde-OH was from Novabiochem. The N-hydroxysuccinimide-ester of Cy5 (Cy5-NHS) was obtained from Amersham. Alexa488- and Alexa633-NHS esters were obtained from Invitrogen/Molecular Probes. Atto488-GTP was purchased from Jena BioScience. All other chemicals were obtained from Fluka at the highest purity available.

Peptide synthesis: Solid phase peptide synthesis of C-terminal peptide **1** (H-CGK$_{(Cy5)}$GHHHHHH-OH) was performed manually using standard Fmoc-deprotection procedures and HBTU-activated couplings.[1;2] The N-terminal cysteine residue was introduced as the N-α-Boc-protected derivative. Cy5-NHS (0.8 equivalents dissolved in dry DMSO) was coupled to the ε-amino group of Lys (16 h at room temperature) after removing the 1-(4,4-dimethyl-2,6-dioxocyclohex-1-yliden)-3-methylbutyl (ivDde)-

group by three 5 min treatments with 3% hydrazine in DMF. In a typical reaction, the labeling was carried out on the solid support with an excess of peptide over amine-reactive dye to ensure complete conversion of the expensive fluorescent dyes. After cleaving the peptide from the resin using a mixture of 33 mg phenol, 25 µL ethanediol, 50 µL H_2O, 50 µL thioanisol and 900 µL trifluoroacetic acid (TFA), it was precipitated with dry ether and washed 3 times, dissolved in water and lyophilized. The peptide was purified on a semi-preparative RP-C8 column with water containing 0.1% TFA and acetonitrile containing 0.08% TFA as mobile phase (yield: 40% (based on crude peptide); purity: >96%). The peptide mass was determined using a Voyager-DE Pro MALDI (Applied Biosystems) and a Finnigan LCQ ESI mass spectrometer. The observed mass was 1826 g/mol (calcd mass: 1826 g/mol). All other fluorescently labeled peptides were synthesized similarly and obtained with a purity of more than 92 %. Molecular weights: H-CGK$_{(Alexa488)}$GHHHHHH-OH - 1703 g/mol (calcd mass: 1702 g/mol); H-CGK$_{(Alexa633)}$GHHHHHH-OH - 2098 g/mol (calcd mass: The structure of Alexa633 has not been released by Invitrogen.). The corresponding mass spectra can be found in supporting figures 4 and 5.

Protein expression: The coding sequence for Ras (H-Ras, aa 1-180) and the RBD (Ras-binding domain of cRaf-1, aa 51-134), respectively, were cloned into the pTXB1-vector (New England Biolabs) and expressed in *E.coli* BL21(DE3) as fusion proteins with the *GyrA* intein from *Mycobacterium Xenopis* and a chitin-binding domain.

For expression of Ypt1 C-terminally truncated by two amino acids and full length MRS6 protein the respective genes were cloned into the pTWIN-1 vector as a C-terminal fusion with an intein (New England Biolabs). Protein expression was induced with IPTG. The cells were harvested by centrifugation and lysed in a M110S Microfluidizer (Microfluids Corporation, Newton). Cell debris and insoluble proteins were removed by centrifugation and the supernatant was loaded onto chitin-beads (New England Biolabs). Proteins were released as their C-terminal thioesters by treatment with 250 mM 2-mercaptoethanesulfonic acid (MESNA) in buffers consisting of 400 mM NaCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, 500 µM GDP at pH 8 for Ras and MRS6 (buffer 1), 150 mM NaCl, 50 mM Tris/HCl, pH 7.4 for the RBD (buffer 2) and 25 mM NaPi, 20 mM NaCl, 0.1 mM MgCl2, pH 7.7 for Ypt1 (buffer 3) for 14 h. Electrospray

mass spectrometry was performed on a Finnigan LCQ system and a Voyager-DE Pro MALDI system (Applied Biosystems).

Ligation: 2 equivalents of fluorescently labeled peptide were added to C-terminal thioester proteins (c = 1.5 mg/ml) and the reaction mixtures were gently shaken for 16 h at 4°C in the dark. Excess peptide was removed by dialysis (5 kDa cut-off membrane) against buffer 1, 2 or 3 for Ras, MRS6, RBD and Ypt1 respectively for 36 h at 4°C. Separation of Ras-, RBD-, Ypt1- and MRS6-thioesters from Ras/Alexa488, Ras/Cy5, RBD/Cy5, RBD/Alexa633, Ypt1/Alexa633 and MRS6/Alexa488, respectively was achieved by Ni-affinity chromatography. Immobilized proteins were released with buffer 1 or 2 containing 250 mM Imidazole. Imidazole was removed by a dialysis step similar to the one described above. Supporting figure 1B shows an SDS-PAGE of RBD/Cy5 after dialysis and Ni-NTA purification but without capture bead treatment. A broad band corresponding to the Cy5-labeled peptide can be identified in the fluorescence scan of the SDS-gel besides the strongly fluorescent band for RBD/Cy5. This small portion of free peptide was not found in samples treated with capture beads. In these cases all proteins showed single bands and only fluorescent bands of the labeled proteins or protein dimers were found (Figure S1 C, D). Additional analysis of the ligation reaction and subsequent purification steps by RP-HPLC indicate the high purity of the reaction product (Figure S2).

Nucleotide exchange: A 5 fold excess of EDTA over Mg^{2+} was used to chelate Mg^{2+} present in the protein buffer. A 50x excess of GppNHp over fluorescent Ras was added to achieve replacement of GDP bound to Ras against GppNHp at 4°C overnight. EDTA and unbound nucleotides are removed by dialysis (24 h) using a 12-14 kDa cut-off membrane. Nucleotide exchange was also performed on Ras proteins using a procedure described previously.[3] The nucleotide content was checked by HPLC at 259 nm using a RP-C18-column (buffer A: 100 mM KP_i, 10 mM TBAB, pH 6.5; buffer B: 100 mM KP_i, 10 mM TBAB, pH 6.5, containing 50% acetonitrile, isocratic 15% buffer B in A).

Fluorescence spectroscopy: Fluorescence spectra were measured with an Aminco-Bowman Series 2 luminescence spectrophotometer (Labsystems) at 25°C in buffer 1, 2 or 3 at concentrations of 500 nM.

Stopped flow measurements: Stopped flow measurements of the RBD/Cy5 – Ras:GppNHp/Alexa488 system were carried out using a Hi-Tech Stopped flow apparatus.

Ras/Alexa488 was excited at 491 nm and the intensity of the FRET signal generated by RBD-Cy5 was monitored through a filter with a cut-off wavelength of 645 nm. Binding of Atto488-GTP to nucleotide free Ras was performed at a concentration of 50 nM nucleotide and an excitation wavelength of 488 nm. The fluorescence emission was observed through a filter with a cut-off wavelength of 495 nm.

Fluorescence cross-correlation Spectroscopy: The experiments were performed on a ConfoCor 2 apparatus (Zeiss) essentially as described previously.[4] The fluorescence emission is collected from a volume element defined by the focal spot of two overlapping laser beams (488 nm and 633 nm) and two pinholes in the image plane of the objective [5;6]. The emission light is split into two channels by means of filters and detected by two separate avalanche photodiodes. Alexa488 and Atto488 labels were detected in one channel with a bandpass 505-550 nm filter, Alexa633 and Cy5-labeled species in the other channel with a longpass 650 nm emission filter. To prevent unstable signals due to surface adsorption, coverslips were preincubated with bovine serum albumine solution (0.2 mg/mL) for 10 min. Excess BSA solution was removed and the sample was applied in a total volume of 10-25 µL. A nonlinear least squares algorithm was used to fit green and red autocorrelation curves (indices "g" and "r") with the FCS model equation for free Brownian diffusion of a single diffusing species,

$$G(t) = G(0) \cdot \left(1 + t / t_{diff}\right)^{-1} \left(1 + t /((z_o / w_o)^2 t_{diff})\right)^{-1/2}$$

where the amplitude $G(0)$ is inversely related to the number of particles in the effective detection volumes ($G_g = 1/N_g$, $G_r = 1/N_r$) and the diffusion time is related to the diffusion coefficient by $t_{diff} = w_o^2/(4D)$. The structure parameter z_o/w_o was fixed at 6. An additional exponential term was included to account for triplet blinking and, in the case of Cy5, a second exponential term to account for isomerization blinking.[7] Cross correlation curves (index "x") were fitted using the same equation without any blinking terms. As a reference, a highly purified ds-DNA oligonucleotide sample double-labeled with Alexa488 and Cy5 (IBA, Göttingen, Germany) was employed. The maximum cross-correlation amplitude obtained was G_x/G_g = 45% relative to the green autocorrelation and Gx/Gr = 68% relative to the red autocorrelation amplitude. The red autocorrelation amplitude was lower than the green, because of the red detection volume being larger than the green (lateral radii: $w_{o(r)} \approx 0.26$ µm, $w_{o(g)} \approx 0.19$ µm). In the case of MRS6, obvious bursts in the fluorescence time trace were excluded from

the computation of the correlation curve by using the raw data reanalysis option of the ConfoCor2 software.

Current FCCS technology is not suited to monitor low affinity binding reactions, because the concentrations of the labeled proteins have to be on the order of the K_d value to obtain detectable amounts of complex. However, too high concentrations lead to exceedingly high particle numbers in the detection volume and saturation of the detectors. As a consequence, the relatively weak interaction between RBD/Cy5 and Ras:GppNHp/Alexa488 ($K_d \approx 300$ nM) could not be monitored . Reducing the size of the detection volume e.g. by using zero-mode waveguides might render FCCS applicable to such protein-protein interactions.[8]

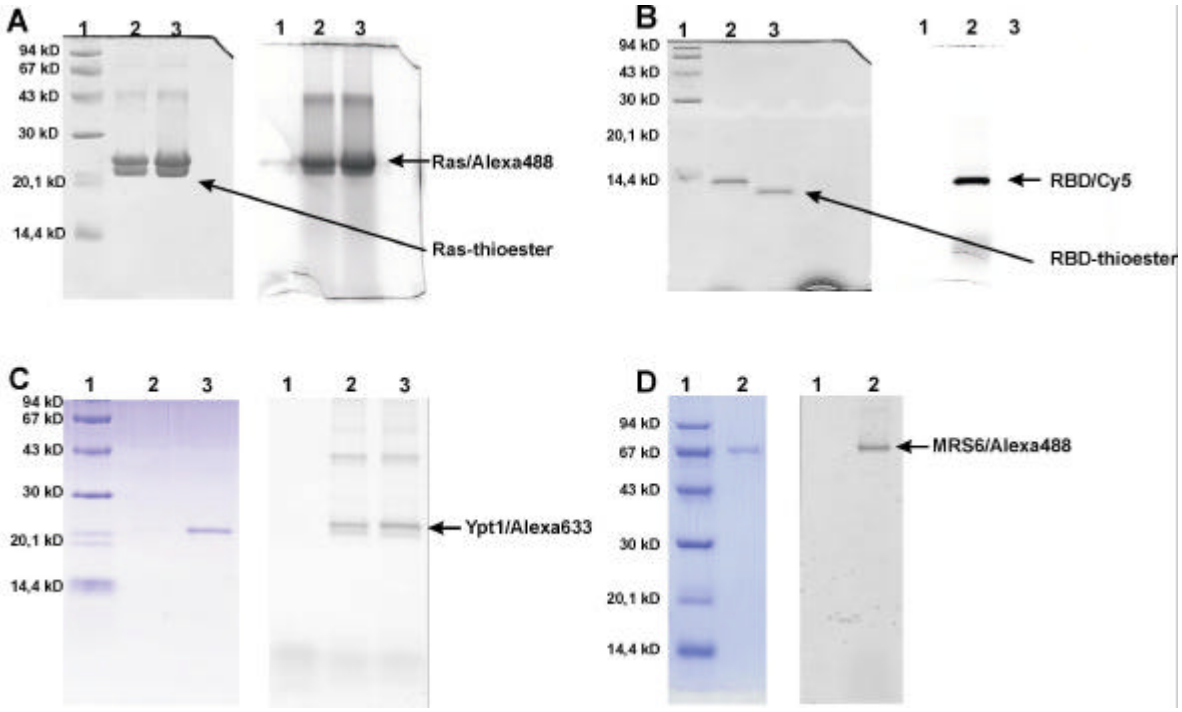

Figure S1: Coomassie stained SDS-PAGE gels and fluorescence scans of C-terminally labeled proteins. A, left) Ras + Alexa488-peptide ligation mixture. Lanes: 1. molecular weight marker, 2. 2 µL reaction mixture Ras/Alexa488 after peptide removal by dialysis, 3. 4µL reaction mixture; A, right) Fluorescence scan; B, left) RBD/Cy5, Lanes: 1. molecular weight marker, 2. 1µL purified RBD/Cy5 (dialysis, capture beads and Ni-NTA), 3. 1 µL of RBD-thioester prior to ligation. B, right) Fluorescence scan; C, left) Ypt1/Alexa633, Lanes: 1. molecular weight marker, 2. 1 µL purified Ypt1/Alexa633, 3. 5 µL purified Ypt1/Alexa633; C, right) Fluorescence scan. D, left) MRS6/Alexa488, Lanes: 1. molecular weight marker, 2. 2 µL purified MRS6/Alexa488; D, right) Fluorescence scan.

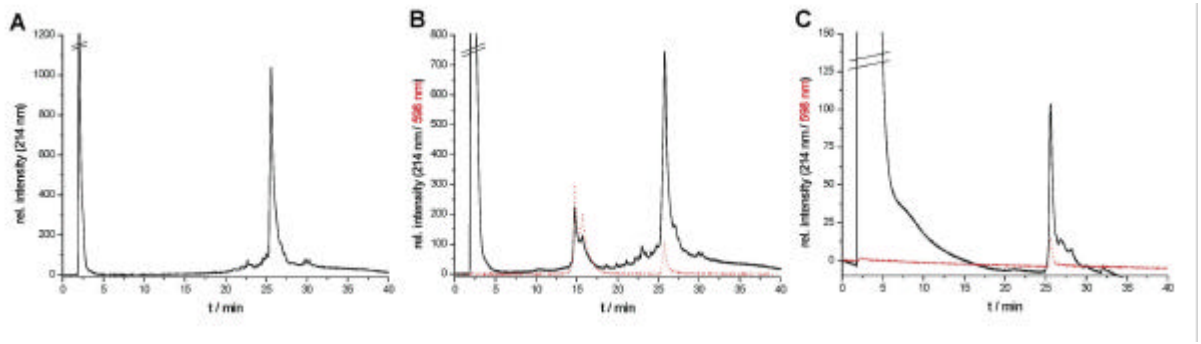

Figure S2: Ras labeling reaction monitored by RP-HPLC using a C4-column with water and acetonitril containing 0.1% TFA as buffer A and B, respectively. A) Ras-Mesna thioester after cleavage of the intein fusion construct and purification on chitin beads. B) Labeling reaction with Cy5-labeled peptide (eluting at 15 min) and Ras thioester. The absorption at 598 nm indicates the Cy5 absorption. The retention time of Ras/Cy5 (26 min) is similar to the one observed for the Ras-thioester. C) Purified Ras/Cy5 after dialysis and treatment with cysteine capture beads. No remaining Cy5-labeled peptide can be detected. The molecular weight was determined by ESI-MS at 22,151 Da (calcd molecular weight: 22,150.6 Da).

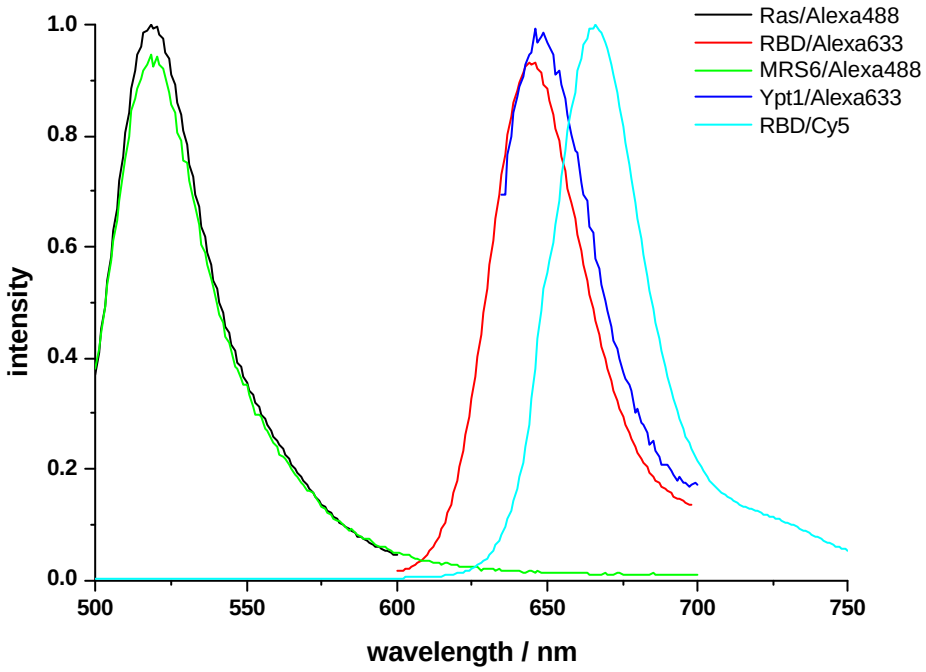

Figure S3: Normalized fluorescence emission spectra of C-terminally labeled pro-teins. Proteins were measured at a concentration of 500 nM in aqueous buffers.

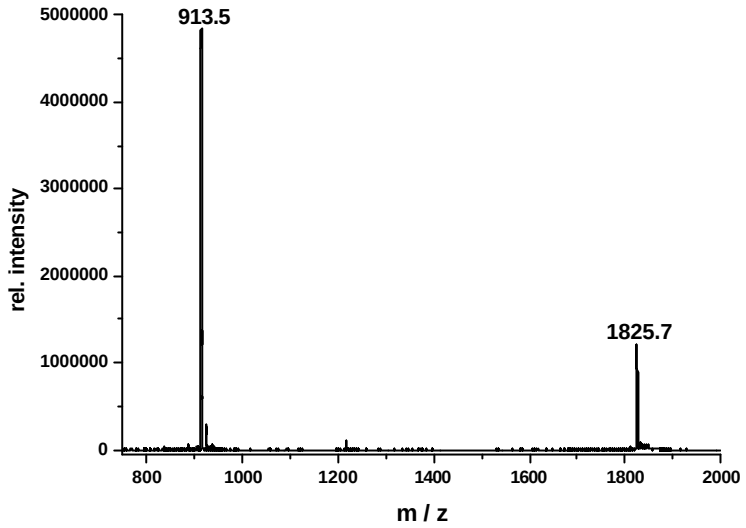

Figure S4: Electrospray mass spectrum of HPLC-purified H-CGK(Cy5)GHHHHHH-OH. Calculated molecular weight: 1,826 g/mol.

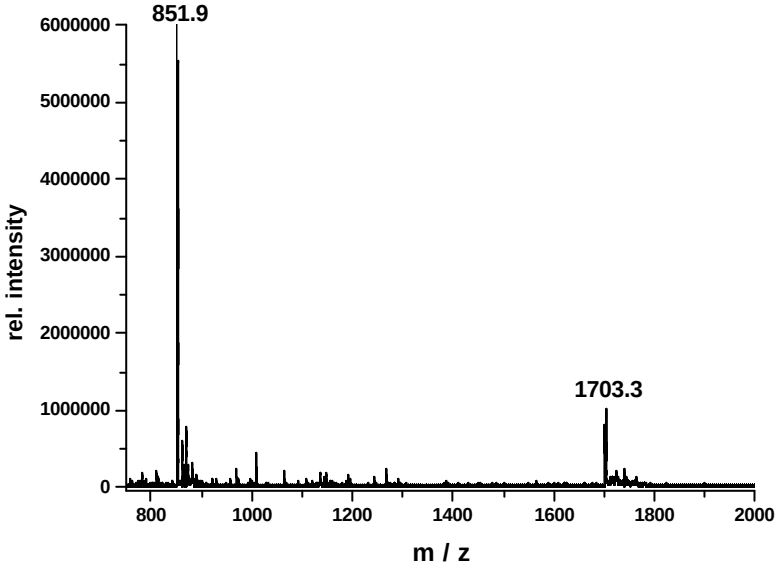

Figure S5: Electrospray mass spectrum of HPLC-purified H-CGK(Alexa488)GHHHHHH-OH. Calculated molecular weight: 1,702 g/mol.

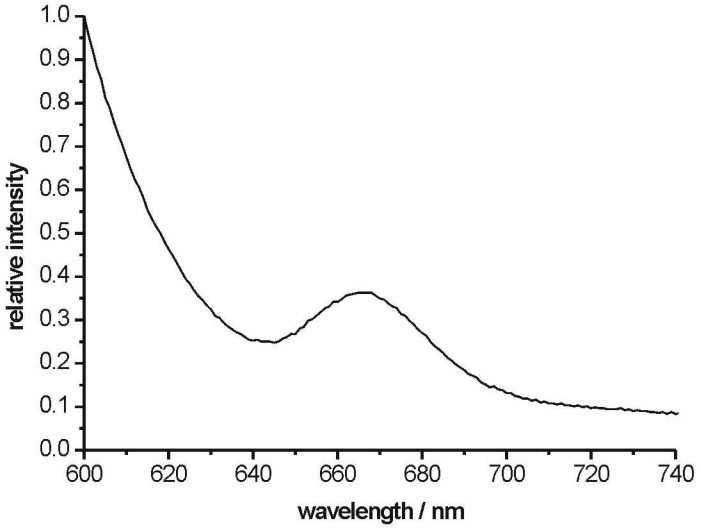

Figure S6: FRET measurement with Ras:GppNHP/Alexa488 and RBD/Cy5 (Ex.: 494 nm, *c* = 500 nM).

[1.] B. Atherton, R. C. Sheppard, *Solid Phase Peptide Synthesis: A Practical Approach*, IRL Press at Oxford University Press, Oxford **1989**.

[2.] M. Schnölzer, P. Alewood, A. Jones, D. Alewood, S. B. H. Kent, *Int.J.Pept.Protein Res.* **1992,** *40*. 180-193.

[3.] J. John, R. Sohmen, J. Feuerstein, R. Linke, A. Wittinghofer, R. S. Goody, *Biochemistry* **1990,** *29*. 6058-6065.

[4.] K. Bacia, I. V. Majoul, P. Schwille, *Biophys.J.* **2002,** *83*. 1184-1193.

[5.] P. Schwille, F. J. Meyer-Almes, R. Rigler, *Biophys.J.* **1997,** *72*. 1878-1886.

[6.] P. Schwille, in *Fluorescence correlation spectroscopy. Theory and applications*Eds.: E. L. Elson, R. Rigler), Springer, Berlin **2001**, p. pp. 360-378.

[7.] J. Widengren, P. Schwille, *Journal of Physical Chemistry A* **2000,** *104*. 6416-6428.

[8.] M. J. Levene, J. Korlach, S. W. Turner, M. Foquet, H. G. Craighead, W. W. Webb, *Science* **2003,** *299*. 682-686.