Measurement of proteases using chemiluminescence-resonance-energy-transfer chimaeras between green fluorescent protein and aequorin

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Homogeneous assays, without a separation step, are essential for measuring chemical events in live cells and for drug discovery screens, and are desirable for making measurements in cell extracts or clinical samples. Here we demonstrate the principle of chemiluminescence resonance energy transfer (CRET) as a homogeneous assay system, using two proteases as models, one extracellular (α -thrombin) and the other intracellular (caspase-3). Chimaeras were engineered with aequorin as the chemiluminescent energy donor and green fluorescent protein (GFP) or enhanced GFP as the energy acceptors, with a protease linker (6 or 18 amino acid residues) recognition site between the donor and acceptor. Flash chemiluminescent spectra (20–60 s) showed that the spectra of chimaeras matched GFP, being similar to that of luminous jellyfish, justifying their designation as

‘Rainbow’ proteins. Addition of the protease shifted the emission spectrum to that of aequorin in a time- and dose-dependent manner. Separation of the proteolysed fragments showed that the ratio of green to blue light matched the extent of proteolysis. The caspase-3 Rainbow protein was able to provide information on the specificity of caspases *in vitro* and *in vivo*. It was also able to monitor caspase-3 activation in cells provoked into apoptosis by staurosporine (1 or 2 μ M). CRET can also monitor GFP fluor formation. The signal-to-noise ratio of our Rainbow proteins is superior to that of fluorescence resonance energy transfer, providing a potential platform for measuring agents that interact with the reactive site between the donor and acceptor.

Key words: apoptosis, bioluminescence, caspase, α -thrombin.

INTRODUCTION

Drug discovery screens, clinical assays and measurements of chemical events in live cells require homogeneous assay systems that do not require a separation step [1,2]. For example, in cell signalling, Ca^{2+} -activated photoproteins and fluors [3,4], and their targeting to organelles [5,6], have revolutionized the study of Ca^{2+} as a cell regulator, now a key target for pharmaceutical intervention. Furthermore, the search for drugs that affect signalling pathways and apoptosis requires intracellular indicators for simultaneous measurement of signals such as Ca^{2+} in more than one subcellular compartment, as well as covalent modification of proteins and protein–protein interactions. We have shown that aequorin and firefly luciferase can be engineered together to measure Ca^{2+} by blue light and ATP by yellow light [7] and that indicators for kinases and proteases can be generated by inserting sites into beetle luciferases [8,9]. However, these were restricted to certain specific recognition sequences. An alternative method was to use a change in the colour of bioluminescence by resonance energy transfer, which occurs in certain luminous coelenterates and some other luminous organisms [1,10–13]. The key parameters are the orientation of the donor and acceptor, and the distance between them, the efficiency depending on the sixth power of the distance separating them. Thus beyond approx. 5–10 nm there is essentially no energy transfer. This means that within a chimaera of aequorin and green fluorescent protein

(GFP), these will be close enough for energy transfer to occur, but on separation the molecules will be too far apart in free solution to allow any significant transfer [1,10]. Chemiluminescence resonance energy transfer (CRET) requires no exciting light source. Thus the only way of exciting the fluor is by energy transfer, resulting in a superior signal-to-noise ratio compared with fluorescence resonance energy transfer (FRET).

Here we report a proof of principle that CRET chimaeric indicators can be used to quantify two model proteases, one important extracellularly (α -thrombin) and the other intracellularly (caspase-3, which is crucial in apoptosis). We also show that these can be used in live cells and we provide a useful potential system for investigating protease specificity *in vitro* and in cells. A particular problem with GFP and with the more recently developed red fluorescent protein from the coral *Discosoma* is that not all the protein molecules fold correctly to become fluorescent [13–16]. Unfolded protein will activate stress responses [17], and will decrease the effectiveness of FRET indicators. We also report here that CRET can be used to monitor GFP fluor formation.

α -Thrombin is a serine-type extracellular protease in the terminal stages of the coagulation cascade [18]. The six-residue α -thrombin substrate site (LVPRGS; single-letter amino acid codes) based on the natural substrate for Factor XIII was engineered between the C-terminus of wild-type GFP or enhanced GFP (EGFP) and the N-terminus of aequorin to create a CRET

Abbreviations used: CCD, charge-coupled device; CRET, chemiluminescence resonance energy transfer; EGFP, enhanced green fluorescent protein (GFP human codon usage, with the mutation S65T); EGCAq, EGFP–caspase-3 linker–aequorin; EGTAq, EGFP– α -thrombin linker–aequorin; FMK, fluoromethylketone; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; GAq, GFP–aequorin; GCAq, GFP–caspase-3 linker–aequorin; GTAq, GFP– α -thrombin linker–aequorin; LAq, firefly-luciferase–aequorin; Z-, benzyloxycarbonyl.

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α -thrombin indicator. Caspases are a family of specific cysteine proteases that participate in an irreversible cascade triggered in response to apoptotic signals [19–22]. The caspase-3 amino acid recognition sequence DEVD was engineered between the C-terminus of GFP or EGFP and the N-terminus of aequorin. It has been reported that the tetrapeptide sequences used in fluorescent and chromogenic indicators for the assay of caspases *in vitro* might not be entirely specific for a particular caspase [23,24]. We show that genetically engineered chimaeras between a bioluminescent donor and fluorescent acceptor, called 'Rainbow' proteins [5,25], can be used to investigate aspects of the specificity of caspases *in vitro* and in live cells.

EXPERIMENTAL

Materials

DNA species encoding wild-type aequorin and GFP were isolated by PCR from freeze-dried jellyfish as described previously [3,5]. Humanized EGFP plasmid DNA was purchased from ClonTech (Basingstoke, Hants., U.K.). Bio-X-Act polymerase and thermal cycler conditions were used in accordance with the manufacturer's instructions (Bioline Ltd, London, U.K.), the annealing temperatures being adjusted for the melting temperatures of each primer pair. The TNT[®] wheatgerm transcription/translation system, the Klenow fragment of *Escherichia coli* DNA polymerase I and amino acids were purchased from Promega (Chilworth, Southampton, U.K.). Deoxynucleoside triphosphates were from Pharmacia (Milton Keynes, U.K.). α -Thrombin (Sigma, Poole, U.K.) was used at final concentrations varying from 3.2 to 500 units/ml. Initially 250 units were dissolved in sodium citrate buffer, pH 6.5, to give a stock concentration of 2500 units/ml, which was used fresh or was stored at -20°C until use. Aliquots of α -thrombin were heat-denatured at 100°C for 20 min for use as a control when testing the effect of α -thrombin on the GFP-caspase-3 linker-aequorin (GCAq) and EGFP- α -thrombin linker-aequorin (EGTAq) Rainbow proteins. PCR amplification products were cleaned (Qiagen, Crawley, West Sussex, U.K.) and ligated into the T7 promoter-controlled vectors, pTARGET (Promega) and Pet3a (Invitrogen, R&D Systems, Abingdon, Oxon., U.K.), in accordance with the manufacturer's instructions. The pLA19 vector containing the DNA species that encode the firefly-luciferase-aequorin (LAq) recombinant protein, was developed by A. S. Ribeiro in our laboratory [5]. Transformation of insert DNA into DH5 α and BL21(DE3) cells (Gibco BRL, Paisley, Renfrewshire, U.K.) were performed by heat shock six times at 37°C and 0°C for 30 s in CaCl_2 (50 mM in 10 mM Tris/HCl, pH 8.0). Transformed bacteria were grown on Luria-Bertani agar plates containing 50 $\mu\text{g}/\text{ml}$ ampicillin and induced where appropriate with 1 mM isopropyl β -D-thiogalactoside. Cell culture reagents and plasticware were obtained from Gibco BRL and Life Sciences International (Basingstoke, Hants., U.K.) respectively. Transfection was performed in accordance with the manufacturer's instructions by using Transfectamine Plus (Gibco BRL) and Fugene6 (Boehringer Mannheim, Germany). HeLa OHIO, COS7 and A549 cells were purchased from ECACC (Porton Down, Salisbury, Wilts., U.K.). Coelenterazine was from Prolume (Beverly Hills, CA, U.S.A.). All other reagents were purchased either from Nycomed Amersham (Little Chalfont, Bucks., U.K.) or Sigma. TNT[®] one-step transcription/translation kit was from Promega. Caspase-3, caspase-9, caspase-3 inhibitor, caspase-9 inhibitor and caspase inhibitor I (benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone) were purchased from Calbiochem (La Jolla, CA, U.S.A.). Caspase-3 was a human recombinant protein expressed in *E. coli*,

with activity 100 units/ μl and a specific activity of 12000 units/ μg of protein. Caspase-3 (5000 units) was diluted in 50 μl of 50 mM Hepes (pH 7.4)/100 mM NaCl/0.1% 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulphonic acid ('CHAPS')/1 mM EDTA/10% (v/v) glycerol/10 mM dithiothreitol to obtain 100 units/ μl of solution. Caspase-9, a human recombinant protein expressed in *E. coli*, was supplied with an activity of 100 units, as a freeze-dried powder, and a specific activity of 400 units/mg of protein. Caspase-9 (100 units) was diluted in 50 μl of the same buffer as used for caspase-3 to obtain 2 units/ μl of solution. Aliquots of both enzymes were prepared and stored at -20°C . Caspase-3 inhibitor II (Calbiochem; catalogue no. 264155) (synonym Z-DEVD-FMK) is a potent cell-permeable and irreversible inhibitor of CPP-32/apopain and a member of the ICE/CED-3 family of cysteine proteases. Caspase-9 inhibitor II [Calbiochem; catalogue no. 218776; synonym LEHD-CHO; (Ac-Ala-Ala-Val-Ala-Leu-Leu-Pro-Ala-Val-Leu-Leu-Ala-Leu-Leu-Ala-Pro-Leu-Glu-His-Asp-CHO)] is a potent cell-permeant and reversible inhibitor of caspase-9 (ICE-LAP6, Mch6). Caspase inhibitor I (Calbiochem; catalogue no. 627610) (synonym caspase-1 inhibitor V) is a highly specific cell-permeant irreversible inhibitor of caspase-1-like proteases, which inhibits Fas-mediated apoptosis in Jurkat T-cells. All the inhibitors were diluted in DMSO (tissue culture grade; Sigma) to obtain a 10 mM stock, then stored at -70°C . Staurosporine (Sigma) was dissolved in DMSO at a stock concentration of 1 mM.

Generation of CRET chimaeras

CRET chimaeras were engineered by two-step PCR [3]. The coding sequences of the primers, prepared with an Applied Biosystems 392 DNA synthesizer and purified as 'trityl off', were as follows (underlining indicates codons for the engineered protease substrate site): GFP4, 5'-TAATACGACTCACTAT-AGGGAGAATGAGTAAAGGAGAAGAAC-3' (5' sense plus T7 promoter at N-terminus of GFP); GFP5, 5'-CCACA-CAGATCTATGAGTAAAGGAGAAGAACTTTTC-3' (5' sense GFP with *Bgl*/II restriction site for subcloning into Pet3a); GFP8, 5'-ATGGATGAACTATACAAAGTCAAGCTTACATCAGAC-3' (5' sense GFP to link to the N-terminus of aequorin); GFP9, 5'-GTCTGATGTAAGCTTGACTTTGTA-TAGTTTCATCCAT-3' (3' anti-sense GFP linking to N-terminus of aequorin); cpp321a, 5'-ATCCACCTCATCCCCAGAGCC-AGAGCCAGAGCCCTTGTACAGCTCGTC-3' (3' anti-sense with caspase linker at the C-terminus of EGFP); cpp321b, 5'-GATGAGGTGGATGGGGGCTCTGGCTCTGGGGTGGT-CAAGCTTACATCA-3' (5' sense with caspase linker to 5' aequorin); cpp321c, 5'-TAATACGACTCACTATAGGGAG-AATGGTGAGCAAGGGC-3' (5' sense plus T7 promoter at the N-terminus of EGFP); cpp321d, 5'-ATCCACCTCATC-CCCAGAGCCAGAGCCAGAGCCCTTTGTATAGTTCA-TC-3' (3' anti-sense caspase linker to C-terminus of GFP); eGFPT, 5'-GGAGCCCCGGGGCACCAGCTTGTACAGCT-CGTCCAT-3' (3' anti-sense with α -thrombin linker at the C-terminus of EGFP); GAL1, 5'-GGAGCCCCGGGGCACCAGCTTGTATAGTTTCATC-3' (3' anti-sense GFP α -thrombin linker at the C-terminus); GAL2, 5'-CTGGTGCCCCGGGG-CTCCGTCAAGCTTACATCA-3' (5' sense α -thrombin linker at the N-terminus of aequorin); PET1, 5'-CCACACAGAT-CTATGGTCAAGCTTACATCA-3' (5' sense aequorin with *Bgl*/II restriction site at 5' end for subcloning into pET3a); PET2, 5'-CCCATCAGATCTTTAGGGGACAGCTCC-3' (3' anti-sense C-terminus of aequorin with *Bgl*/II restriction site at C-terminus for subcloning into Pet3a); A5, 5'-TAAT-

ACGACTCACTATAGGGAGAATGGTCAAGCTTAC-ATCAGACTTCGAC-3' (5' sense with T7 promoter at the N-terminus of aequorin).

DNA species coding for aequorin (189 residues) and for wild type or enhanced GFP (238 residues) (Promega) were used as targets for PCR amplification. Oligonucleotides GFP4 and cpp321c incorporated the T7 RNA polymerase promoter sequence at the 5' end of GFP and EGFP respectively. The oligonucleotide pairs for PCR used were as follows: 1. To make the GAq (GFP-aequorin), a first stage PCR was performed using GFP4 (sense) and GFP8 (antisense) primers with GFP, and GFP8 (sense) and PET2 (antisense) primers with aequorin, followed by second stage PCR to join the two cDNAs generated in the first stage with GFP4 (sense) and PET2 (antisense) primers; 2. To make the GTAq (GFP- α -thrombin linker-aequorin), a first stage PCR was performed using GFP4 (sense) and GAL1 (antisense) primers with GFP, and GAL2 (sense) and PET2 (antisense) with aequorin, then a second stage PCR was performed to join the two cDNAs generated in the first stage using GFP4 (sense) and PET2 (antisense); 3. To make the EGTAq (EGFP- α -thrombin linker-aequorin), a first stage PCR was carried out using GFP cpp321c (sense) and GAL1 (antisense) primers with GFP, and GAL2 (sense) and PET2 (antisense) primers with aequorin, followed by a second stage PCR to join the two cDNAs generated in the first stage using cpp321c (sense) and PET2 (antisense); 4. To make the EGCAq (EGFP-caspase linker-aequorin), first stage PCR primers, GFP cpp321c (sense) and cpp321a (antisense), were used with aequorin, followed by a second stage PCR to join the cDNAs generated in the first stage using the primers cpp321b (sense) and PET2 (antisense); 5. To make aequorin alone, primers A5 and PET2 were used with aequorin DNA. The final protein chimaeras were GTAq (GFP-LVPRGS-aequorin), GCAq (GFP-GSGSGSGDEVGGSGSGV-aequorin), EGTAq (EGFP-LVPRGS-aequorin) and EGCAq (EGFP-GSGSGSGDEVGGSGSGV-aequorin).

Formation and assay of the Rainbow protein

Synthesis of the engineered proteins *in vitro* was performed with the transcription-translation wheatgerm cell-free system or by expression in *E. coli* [BL21(DE3)], COS7, A549 or HeLa OHIO cells [3,5,7]. Extracted protein was partly purified by precipitation with $(\text{NH}_4)_2\text{SO}_4$ and stored at -20°C prior to assay.

Extraction and reconstitution of protein chimaeras from cells

Total cell extracts were from COS7 cells expressing EGCAq, EGTAq and LAq. COS7 cells were split at 2×10^5 cells per well in six-well plates; 24 h later, cells were transfected with pEGCA, pEGTA and pLA19 plasmid DNA. At 48 h after transfection, cells were washed twice with ice-cold PBS and extracted with aequorin extraction buffer (400 μl per well). A pool was made with all the extracts from each well for each kind of recombinant protein; each was checked for aequorin activity with the luminometer and 50 μl aliquots were made and stored at -70°C . Reactivation of aequorin with coelenterazine was performed as described previously [3,5,7]. In brief, samples (75 μl final volume, including 60 μl of cell extract) were incubated for 1 h at room temperature in the dark with an equal volume of aequorin reactivation medium (75 μl) [10 mM Tris/HCl (pH 7.5)/500 mM NaCl/1 mM EDTA/5 mM 2-mercaptoethanol/0.1% gelatin] plus 2 μM coelenterazine (Prolume, Beverly Hills, CA, U.S.A.).

Approximately 10^6 cells were extracted from each well in 400 μl ; as described above, pools with the cells expressing the same kind of chimaera were made and aliquots were stored at

-70°C . For each experimental condition, 60 μl of cell extracts was used, which was equivalent to 1.5×10^5 cells. From those, 4×10^4 and 2×10^4 cells were used to determine the chemiluminescent spectra and CRET ratiometric measurements respectively.

Assay of aequorin chemiluminescence

Reactivated protein extract (10 μl) was added to 90 μl of aequorin assay buffer [200 mM Tris/HCl (pH 7.4)/0.5 mM EDTA] and the total chemiluminescent count in 10 s was taken as the background luminescence. Then 50 mM CaCl_2 was injected to a final concentration of 25 mM; the total chemiluminescent count in a further 10 s was taken as the aequorin activity. Chemiluminescence was measured with a Berthold luminometer or a three-microchannel plate photon-counting intensified charge-coupled device (CCD) imaging camera [3,5,7] (Photek Ltd, St. Leonards, East Sussex, U.K.) (385 pixels $\times$ 288 pixels at 50 Hz for spectra and 512 pixels $\times$ 512 pixels at 60 Hz for ratiometric emissions).

Measurement of chemiluminescent spectra

Spectral measurements were performed with a photon-counting intensified CCD imaging camera (385 pixels $\times$ 288 pixels at 50 Hz; Photek) [3,5]. A 40 μl sample of the reactivated protein was placed in a cuvette containing 60 μl of assay buffer. Immediately after measurement of the background for 20 s, 100 μl of 50 mM CaCl_2 was injected and the total light emission was measured for a further 40 s. The light emission was transformed into a spectrum by using a diffraction-grating monochromator with the input slit at 2 mm. The monochromator was set at 450, 500 or 550 nm to obtain the maximum part of the spectrum. The spectrum was then displayed with the use of the imaging camera, by transmitting the light to the photocathode through a fibre-optic bundle (2 cm in diameter with each individual fibre being 6 μm in diameter). The image data were recorded at video rate (50 Hz). An integrated image was then generated over the 40 s after the addition of CaCl_2 . The visual spectrum was converted by the Photek camera software into data for insertion into a spreadsheet. The spectra were calibrated with either a phosphorescent green-emitting star or a 'white' chemiluminescent light stick as light source, and narrow bandpass filters from 400 to 650 nm (10 nm bandwidth). This generated a simple linear equation enabling the abscissa to be converted into wavelength. This novel application of an imaging camera enabled spectra to be measured from chemiluminescent and bioluminescent sources that flashed for just a few seconds or glowed for many minutes. This would have been impossible with conventional spectrometers. This method is superior to arrays of photodiodes because these are less sensitive. However, considerably more total light emission is required than when performing simple ratiometric measurements. Raw data are presented that have not been smoothed. The spectra were therefore sometimes noisy (compare Figures 2c and 2f), depending on how much activity it was possible to extract from the cells. Nevertheless, it was still possible to make clear conclusions from all the spectral data.

Measurement of CRET

CRET was assayed in cell extracts: 20 μl of reactivated sample from cells extract was added to 80 μl of assay buffer in a 96-well plate and the background was measured for 20 s; 100 μl of 50 mM CaCl_2 was then injected and the light emitted was measured for 40 s. Ratiometric chemiluminescence emissions were obtained with a specially constructed three-microchannel

plate photon-counting intensified CCD imaging camera (Photek) (512 pixels $\times$ 512 pixels at 60 Hz), set up to quantify up to four colours simultaneously. Light from the sample passed through either two calibrated interference filters (Melles Griot, Irvine, CA, U.S.A.) with bandwidths of 10 nm (wavelength 450.0, bandwidth 10 ± 2 nm, part no. 03 FIV 004; wavelength 500.0, bandwidth 10 ± 2 nm, part no. 03 FIV 006), 40 nm (wavelength 450.0, bandwidth 40 ± 8 nm, part no. 03 FIV 028; wavelength 500.0, bandwidth 40 ± 8 nm, part no. 03 FIV 038) or 80 nm (wavelength 450.0, bandwidth 80 ± 16 nm, part no. 03 FIB 004, wavelength 500.0, bandwidth $80 \pm 16/-20$ nm, part no. 03 FIB 006), routinely with peak emissions at 453.3 nm (bandwidth 36.0) and 503.0 nm (bandwidth 39.2) respectively, or a beam-splitter (MultiSpec Micro-Imager; Optical Insights, Santa Fe, NM, U.S.A.) with up to four interference filters with a bandwidth of 20 nm. The advantage of using the 40 nm bandwidth filters was their greater sensitivity than the 10 or 20 nm bandwidth filters, which resulted in some 80–90% loss of total light emission. Ratios depended on the construct and the precise filters, showing that a wide bandpass can be used for CRET.

Chemiluminescence and bioluminescence indicators generate a much lower absolute photon emission than their fluorescent counterparts. There are two reasons for this. First, the chemiluminescent substrate (luciferin in the case of bioluminescence) reacts only once, whereas fluors can be excited many times. Secondly, the protein catalysts (luciferases and apophotoproteins) of chemiluminescent reactions are poor enzymes, with catalytic-centre activities usually in the range 0.1–4 [1]. In fluorescence, it is often necessary to use filters with a relatively narrow bandpass (typically 10 nm) to obtain a good signal-to-noise ratio. To optimize photon detection for our chemiluminescent indicators, we investigated whether it was possible to use filters with a wider bandpass. Filters with bandpasses of 10, 20, 40 and 80 nm were compared. CRET measured with filters with a 80 nm bandpass detected five times more light than with a filter with a 20 nm bandpass. However, emission ratios at 500 and 450 nm were usable with all filters. Filters with a 20 nm bandpass were used with the beam splitter; 5 cm filters with a 40 nm bandpass were used in a specially constructed slide in front of the Photek imaging camera. Although the absolute ratios of 'green' to 'blue' light measured at 500 and 450 nm depended on which filter pair was used (compare Figures 2, Figures 4–6 and Table 2), the fractional difference in ratio at 500 and 450 nm comparing a chimaera with free aequorin was quite similar, showing that either could be used with good sensitivity. However, this also resulted in significant differences in the ratios. For example, the CRET caspase chimaera (EGCAq) produced a maximum ratio of 12 between 500 and 450 nm with the 40 nm bandwidth filters, and a ratio of 6 between 500 and 450 nm with the 20 nm bandwidth filters. It was somewhat surprising that a larger ratio was obtained with the wider bandpass filters because these would have been expected to result in a larger spectral overlap. The reason was that the emission peaks of the CRET chimaeras were closer to 510 nm than 500 nm and the 40 nm bandpass filters were able to collect more photons in the green region of the CRET spectrum.

These results show that, for full optimization of CRET ratios at any two given wavelengths, a range of filters with different peak transmissions and bandpasses need to be compared.

Demonstration of proteolysis of the Rainbow proteins

Proteolysis of the respective CRET chimaeras was demonstrated by Western blotting after PAGE; the proteolysed fragments containing EGFP or aequorin were detected with an antibody

specific for either GFP or aequorin. The correct molecular masses of the CRET recombinant proteins and their proteolysed products were demonstrated by SDS/PAGE followed by densitometric analysis [3,5]. Samples (100 μ g) of the total cell extract containing the chimaeric CRET proteins (EGCAq or EGTAq) were incubated with 150 units/ml caspase-3 or 50 units/ml α -thrombin respectively for 0, 2 and 4 h. Control reactions were performed with the addition of 150 i.u./ml caspase-3 plus 10 μ M caspase-3 inhibitor, or denatured α -thrombin. In the caspase-3 proteolysis, LAq protein was used as negative control; in α -thrombin proteolysis, EGCAq was used as negative control. Reactions were stopped by the addition of an equal volume of $2 \times$ SDS/PAGE loading buffer and boiling at 100 °C for 10 min; 50 μ g of total proteins from each reaction was loaded on a 12.5% (w/v) polyacrylamide gel and SDS/PAGE was performed. The proteins were then transferred to a nitrocellulose paper and left blocking overnight at 4 °C with 5% (v/v) milk in TBS-T (Tris-buffered saline containing Tween 20). Membranes were then incubated with the primary antibodies. Rabbit polyclonal antibody against GFP (a gift from Dr David H. Llewellyn), or rabbit polyclonal antibody against aequorin (prepared by us) was used in 1:3000 dilutions. The blots were washed three times with TBS-T for 10 min. The secondary antibody, goat anti-rabbit IgG (H+L)-horseradish peroxidase conjugate antibody (Bio-Rad, Hemel Hempstead, Herts., U.K.) (1:6000 dilution) was then added. The blots were washed three times with TBS-T for 10 min. The horseradish peroxidase was detected with a chemiluminescence kit (Pierce, Rockford, IL, U.S.A.). The relative amounts of the fragment bands were correlated with the ratio of light emission at 500 and 450 nm.

Apoptosis

Apoptosis was observed microscopically on typical condensed nuclei, and confirmed with fluorescence microscopy [24] after staining with the dye Hoechst 3342 (10 μ g/ml in standard PBS).

RESULTS

Generation and spectral measurement of CRET constructs

Synthesis of CRET chimaeric proteins was confirmed by a single DNA band of the predicted size; the correct molecular mass of recombinant protein was confirmed by SDS/PAGE followed by densitometric analysis [3,5]. CRET was demonstrated first by measuring the chemiluminescence spectrum of chimaeras, assessing the peak emission and bandwidth and whether there was any evidence for a bimodal spectrum. Flash spectra (Figure 1) showed that both GTAq and GFP-caspase-3 linker-aequorin (GCAq) had spectra shifted towards the green compared with aequorin alone, with a peak emission similar to that of GFP fluorescence (Table 1) [13,15]. The peak emission and bandwidth of the emission spectra were similar to those reported for GFP fluorescence and emission from the live jellyfish *Aequorea* [12]. The validity of using the imaging camera to quantify bioluminescent spectra was confirmed by the spectra of *Vargula* luciferase and a live glow-worm (Table 1), which closely matched those reported previously [1]. Wild-type aequorin, being a mixture of at least five isoaequorins, had a slightly different flash spectrum from the recombinant photoprotein (Figure 1a). No significant spectral shifts were found when GFP and aequorin were in free solution, not linked together as a chimaera, under the conditions of these experiments (results not shown). The addition of aequorin to either GTAq (EGTAq) or GCAq (EGCAq) chimaeras generated a bimodal spectrum, the second peak corresponding to free aequorin (A. Bermúdez Fajardo, un-

Figure 1 Chemiluminescence spectra of CRET constructs

Flash chemiluminescence spectra, taken with the monochromator setting at 450 nm, were obtained as described in the Experimental section. Results are means for three determinations over a time of 40 s after the addition of 25 mM Ca^{2+} (final concentration) to the aequorin in 200 mM Tris/0.5 mM EDTA pH 7.4. (a) Wild-type and recombinant aequorin; (b) recombinant aequorin and GCAq (GCA); (c) recombinant aequorin and GTAq (GTA).

published work). There was no evidence of any significant spectral bimodality with the GCAq chimaera, although a small peak was observed with GTAq, suggesting that a small proportion of the GTAq chimaeras had not fully formed the GFP fluor. The small differences in bandwidths between GCAq, GTAq, EGTAq and EGCAq were reflected in the emission ratios at 500 and 450 nm. The differences in emission ratios between GTAq and GCAq, and between EGTAq and EGCAq, were not dependent on the bandpass of the filters used to obtain the CRET emission ratio. They seemed to be inherent in the construct, not due to differences in GFP fluor formation. GFP–aequorin, with no linker, resulted in some energy transfer, but this was much weaker than when a linker was present, the ratio at 500 and 450 nm being less than twice that of free aequorin.

Although larger amounts of active protein were obtained after transfecting cells with the EGFP constructs EGTAq and EGCAq,

Table 1 Emission peaks and bandwidths

Spectra were obtained as described in the Experimental section. The bandwidth is the spectral width at half-maximum emission. Data for *Aequorea*, GFP and EGFP were obtained from the references shown. Values are means $\pm$ S.E.M. for three observations, except published ones.

Bioluminescent protein	Peak emission (nm)	Bandwidth (nm)
Wild-type aequorin	464.7 $\pm$ 1.9	94.1 $\pm$ 3.2
Recombinant aequorin	484.0 $\pm$ 3.0	78.9 $\pm$ 5.2
GTAq	503.9 $\pm$ 7.5	68.5 $\pm$ 4.3
EGTAq	504.3 $\pm$ 0.82	30.7 $\pm$ 1.6
GCAq	507.1 $\pm$ 0.3	57.3 $\pm$ 1.5
EGCAq	504.7 $\pm$ 0.7	27.3 $\pm$ 1.2
<i>Aequorea victoria</i> [11]	508	30
GFP [14]	508	30
EGFP ([12,14], and Clontech catalogue)	508	30
<i>Vargula</i> luciferase	461.5 $\pm$ 2.8	77.7 $\pm$ 2.0
Live glow-worm, <i>Lampyrus noctiluca</i>	550.5 $\pm$ 7.2	62.7 $\pm$ 2.5
Green phosphorescent star	518.8 $\pm$ 7.2	95.6 $\pm$ 2.7

and more fluorescent cells were seen under the microscope than with chimaeras with GFP, there were no major differences in their spectra. Although the EGFP chimaeras had a slightly narrower bandwidth, this was consistent with a better formation of the fluor in the EGFPs [13–15], and also suggested that the 390 nm excitation peak in GFP, which is absent from EGFP (the GFP-S65T mutant), was not important for energy transfer.

Effect of α -thrombin and caspase-3 on EGTAq and EGCAq Rainbow proteins

The ability of CRET to monitor proteolysis was assessed initially by measuring the effect of the proteases α -thrombin and caspase-3 respectively on the spectrum of individual constructs (Figure 2). Both α -thrombin (50 units/ml) and caspase-3 (5000 units/ml) incubated for 3 h at room temperature with EGTAq and EGCAq respectively resulted in a change in the emission spectral peak from that of the GFP to that of aequorin (Figures 2d and 2b). The effect of α -thrombin was destroyed if it was first denatured by heat. Inclusion of the caspase-3 inhibitor (10 μ M) prevented the spectral shift (Figure 2c). A spectral change was easily detectable down to 150 units/ml caspase-3. The specificity of the effect of protease was confirmed by the fact that no significant spectral change was observed when caspase-3 was added to the α -thrombin Rainbow indicator (EGTAq) (Figure 2e). Similarly, no significant spectral change was observed when α -thrombin was added to the caspase-3 Rainbow indicator (EGCAq) (Figure 2a). There was also no detectable effect of the proteases on the emission spectrum of LAq (Figure 2f), our standard cytosolic indicator for Ca^{2+} and ATP (Figures 2f and 2g).

Proteolysis of EGCAq in these experiments was confirmed by separation of the cleavage products by SDS/PAGE under reducing conditions, detecting GFP and aequorin with their respective specific antibodies (Figure 3). LAq controls showed no band with anti-GFP antibody (Figure 3A, lane 1), but a strong band at approx. 83 kDa (62 kDa for firefly luciferase plus 21 kDa for aequorin) was observed with the anti-aequorin antibody (Figure 3B, lane 1). No additional bands were observed after incubation with caspase-3 or with caspase-3 plus inhibitor (Figures 3A and 3B, lanes 2 and 3). Rainbow protein EGCAq produced a strong band at approx. 50 kDa (28 kDa for GFP + 21 kDa for aequorin + 2.16 kDa for the linker) with both anti-GFP and anti-aequorin antibodies (Figures 3A and 3B, lanes 4–9). Even at zero time a small amount of cleavage had

Figure 2 Effect of α -thrombin and caspase-3 on CRET spectra

Total cell extracts from COS7 cells containing the EGCAq (a–c), EGTAq (d, e) and LAq (f, g) chimaeric proteins (60 μ l) were incubated for 3 h at room temperature with 50 units/ml α -thrombin (a, d, f) or 5000 units/ml caspase-3 (b, e, g). Negative controls were performed in the absence of α -thrombin or caspase-3 or in the presence of caspase-3 plus 10 μ M of caspase-3 inhibitor (c). Aequorin reactivation and flash chemiluminescence spectra (with the monochromator setting at 450 nm) were performed as described in the Experimental section. Control and experimental spectra are represented by heavy and faint lines respectively.

occurred, as shown by the appearance of two faint bands at 28 kDa with the anti-GFP antibody (Figure 3A, lane 4) and at 21 kDa with the anti-aequorin antibody (Figure 3B, lane 4). However, incubation with caspase-3 clearly resulted in a substantial increase in both the GFP (Figure 3A, lanes 6 and 8) and aequorin bands (Figure 3B, lanes 6 and 8), there being more after 4 h than after 2 h. Furthermore, the appearance of these bands was markedly inhibited by the presence of the caspase-3 inhibitor (10 μ M) (Figures 3A and 3B, lanes 7 and 9). Under these conditions the cleavage of the Rainbow chimaera was not complete, being approx. 50%. This correlated well with an approx. 50% decrease in the emission ratio of EGCAq at 500 and 450 nm under these conditions. As can be seen from Figure 3, some other degradation products were detected with both the anti-GFP and anti-aequorin antibodies (lanes 4–9, approx. 30 kDa). The intensity of this intermediate band seemed to be increased by the addition of the caspase-3 inhibitor (Figures 3A and 3B, compare lanes 7 and 9 with lanes 6 and 8).

EGTAq proteolysis was confirmed also by separation of the cleavage products by SDS/PAGE, by using the same specific antibodies against GFP and aequorin (Figures 3C and 3D). EGCAq controls showed a strong band at approx. 49 kDa with anti-GFP and anti-aequorin antibodies (Figures 3C and 3D, lane 1). However, unlike EGTAq, with EGCAq the bands of EGFP (28 kDa), aequorin (21 kDa) and degradation products (approx. 30 kDa) were observed before and after incubation with α -thrombin or denatured α -thrombin (Figures 3C and 3D, lanes 1–3). This indicated that some cleavage of EGCAq had occurred that was not the result of incubating EGCAq with α -thrombin. Similar results were obtained with EGCAq at time zero and after incubation of EGCAq with caspase-3 or caspase-3 plus inhibitor (Figures 3A and 3B, lanes 4–9). Rainbow protein EGTAq produced a strong band at approx. 49 kDa (28 kDa for GFP plus 21 kDa for aequorin plus 0.72 kDa for the linker) with both anti-GFP and anti-aequorin antibodies (Figures 3C and 3D, lanes 4, 5, 7 and 9). Under these conditions, incubation with α -thrombin

Figure 3 Demonstration of proteolysis of CRET constructs by SDS/PAGE

Total protein extracts from COS7 cells expressing EGTAq (EGTA) and EGCAq (EGCA) Rainbow proteins were used to demonstrate the proteolysis of these CRET constructs. Total protein (100 μ g) containing the EGCAq or EGTAq was incubated for 0, 2 and 4 h in the presence of 150 units/ml caspase-3 or 50 units/ml α -thrombin respectively. Negative controls were performed by incubation for the same period with caspase-3 plus 10 μ M caspase-3 inhibitor for EGCAq, or with denatured α -thrombin for EGTAq. LAq (LA) and EGCAq were used as controls for caspase-3 and α -thrombin respectively. Proteolysis reactions were stopped by the addition of an equal volume of 2 $\times$ SDS/PAGE loading buffer and incubated for 10 min in boiling water. Samples were separated by SDS/PAGE [12.5% (w/v) gel] and the products of caspase-3 and α -thrombin proteolysis were identified by GFP-specific and aequorin-specific antibodies as described in the Experimental section. Abbreviation: DP, degradation products. **(A, C)** Anti-GFP rabbit polyclonal antibody (1:3000 dilution); **(B, D)** anti-aequorin rabbit polyclonal antibody (1:3000 dilution). **(A)** and **(B)** correspond to the caspase-3-dependent proteolysis of EGCAq: lane 1, LAq control (no treatment), 4 h; lane 2, LAq plus caspase-3, 4 h; lane 3, LAq plus caspase-3 plus caspase-3 inhibitor; lanes 4, 6 and 8, EGCAq plus caspase-3, incubated for 0, 2 and 4 h respectively; lanes 5, 7 and 9, EGCAq plus caspase-3 plus inhibitor, incubated for 0, 2 and 4 h respectively. **(C)** and **(D)** correspond to the α -thrombin-dependent proteolysis of EGTAq: lane 1, EGCAq control (no treatment), 4 h; lane 2, EGCAq plus α -thrombin, 4 h; lane 3, EGCAq plus denatured α -thrombin (dThrombin), 4 h; lanes 4, 6 and 8, EGTAq plus α -thrombin, incubated for 0, 2 and 4 h respectively; lanes 5, 7 and 9, EGTAq plus α -thrombin, incubated for 0, 2 and 4 h respectively.

clearly resulted in the almost total proteolysis of EGTAq into its two products EGFP and aequorin, because both EGFP (Figure 3C, lanes 6 and 8) and aequorin (Figure 3D, lanes 6 and 8) bands are visible and more EGTAq was degraded after 4 h than after 2 h. Furthermore these bands did not appear when EGTAq was incubated for the same period with denatured α -thrombin (Figures 3C and 3D, lanes 7 and 9). Under these conditions the cleavage of the Rainbow chimera at 4 h was almost complete and correlated well with the decrease in the emission ratio of EGTAq at 500 and 450 nm under these conditions (Table 2). In contrast with the EGCAq gels, there were no additional minor bands, confirming that the bands with EGCAq were due to additional cleavage by caspases in the cells.

These proteolysis experiments also enabled us to compare the apparent quantum yield of free aequorin with that of aequorin emission from GFP. There was a 20% difference in total light emission from EGCAq and from EGTAq compared with the emission when these chimaeras had been completely cleaved to form GFP plus aequorin; the spectrum after cleavage was the same as that of free aequorin. This confirmed that there was

energy transfer from aequorin to GFP, but that it had no influence on chemiluminescence quantum yield.

Effect of caspase-3 and caspase-9 on GCAq *in vitro*

It has been reported that four amino acids are sufficient for a recognition site for several caspases [22,23]. For example, the site for caspase-3 is DEVD and that for caspase-9 is LEHD. These form the basis of fluorescent indicators that are commercially available and were the basis of constructing the Rainbow protein indicator for caspase-3 reported here. To test the specificity of this for caspase-3, EGCAq, extracted from COS-7 cells as described in the Experimental section, was incubated for 3 h with 5000 units/ml caspase-3 or 150 units/ml caspase-9, with or without their respective inhibitors at 10 μ M (Figure 4). The results show that both caspase-3 and caspase-9 at the doses used were able to cut the EGCAq Rainbow protein, decreasing the ratio of light emission at 500 and 450 nm to that of free aequorin (compare Figures 4B and 4E with Figure 4A). However, some specificity was shown in the presence of the inhibitors. The

Table 2 Effect of proteases on CRET constructs

Total cell extract from COS7 cells containing the EGCAq, EGTAq and LAq chimaeric proteins (60 μ l) were incubated for 3 h at room temperature with 50 units/ml thrombin or with 150 or 5000 units/ml caspase-3. Negative controls were performed in the absence of thrombin or caspase-3, or with the addition of 10 μ M caspase-3 inhibitor. Aequorin reactivation and CRET ratiometric measurements were performed as described in the Experimental section. Values are means $\pm$ S.E.M. for three observations.

Addition	Emission ratio at 500 and 450 nm		
	LAq	EGTAq	EGCAq
None (control)	0.64 $\pm$ 0.02	2.59 $\pm$ 0.25	2.88 $\pm$ 0.04
Thrombin (50 units/ml)	0.65 $\pm$ 0.05	0.68 $\pm$ 0.02	2.92 $\pm$ 0.12
Caspase-3 (150 units/ml)	0.69 $\pm$ 0.00	2.87 $\pm$ 0.14	1.59 $\pm$ 0.14
Caspase-3 (5000 units/ml)	0.36 $\pm$ 0.13	—	0.36 $\pm$ 0.13
Caspase-3 (5000 units/ml) + 10 μ M inhibitor	0.69 $\pm$ 0.00	—	3.67 $\pm$ 0.00

Figure 4 Effect of caspase-3 and caspase-9 (in the presence or absence of inhibitors of caspase-3 and caspase-9) on EGCAq CRET *in vitro*

CRET ratiometric measurements were obtained as described in the Experimental section. Total cell extracts containing EGCAq were incubated in the absence (Control) or presence of 5000 units/ml caspase-3 or 150 units/ml caspase-9, with or without 10 μ M caspase-3 inhibitor (inh.) or caspase-9 inhibitor, for 3 h at room temperature. Results are means $\pm$ S.D. for three determinations.

caspase-9 inhibitor abolished the effect of caspase-9 on the decrease in light emission ratio at 500 and 450 nm (compare Figures 4F and 4E), but had only a very small effect on the caspase-3-induced decrease in emission ratio at 500 and 450 nm (compare Figures 4D and 4B). In contrast, the caspase-3 inhibitor was less specific, causing a marked inhibition of the decrease in emission ratio at 500 and 450 nm with both caspase-3 (compare Figures 4C and 4B) and caspase-9 (compare Figures 4G and 4E). Therefore these results show that these four amino acid sequences were not specific for individual caspases and that the Rainbow proteins can be used to examine this.

Effect of staurosporine on EGCAq in live cells

Staurosporine (1 or 2 μ M) induced apoptosis, detected by fluorescence microscopy (see the Experimental section), in transfected HeLa OHIO cells expressing the EGCAq variant containing the caspase-3 linker, resulting in a time-dependent decrease in the CRET ratio at 500 and 450 nm, 2 μ M being more potent than 1 μ M (Figure 5). The caspase inhibitor Z-VAD-FMK (10 μ M) [19,20] prevented staurosporine-induced apoptosis and inhibited the change in the CRET ratio at 500 and 450 nm (Figure 5). This showed that the caspase-3 proteolytic site DEVD between GFP and aequorin was fully accessible in

Figure 5 Effect of staurosporine on the caspase CRET indicator in live cells

HeLa OHIO cells expressing the caspase-3 indicator EGCAq were incubated for 24 h in tissue culture medium with 1 μ M ($\square$) and 2 μ M ($\circ$) staurosporine, a potent inhibitor of protein kinases, including protein kinase C. To show that staurosporine activity alone had activated the caspase-3 proteolysis of the EGCAq indicator, Z-VAD-FMK (10 μ M), a caspase inhibitor, was added: $\blacktriangle$, 1 μ M staurosporine plus 10 μ M Z-VAD-FMK; $\times$, 2 μ M staurosporine plus 10 μ M Z-VAD-FMK. Control cells were incubated in the absence of staurosporine ($\blacklozenge$). Examination of the cells microscopically showed that most cell nuclei had a typical apoptotic appearance within 24 h. Aequorin was reactivated as described in the Experimental section. A sample of the reactivated cell extract was added to 200 mM Tris/HCl (pH 7.4)/0.5 mM EDTA; Ca^{2+} (25 mM final concentration) was added to activate aequorin chemiluminescence. The emission ratio was monitored at 500 and 450 nm by using the beam splitter. Results are means $\pm$ S.D. for three determinations.

live cells. The effect of the inhibitor was more marked with 1 μ M staurosporine, suggesting that the inhibition was competitive. No significant change in emission ratio at 500 and 450 nm was observed in control cells expressing EGCAq without the addition of staurosporine. Similar effects of staurosporine and the inhibitor were seen when the EGCAq Rainbow protein was expressed in A549 cells (results not shown).

Time- and dose-dependent effect of α -thrombin addition on CRET in EGTAq

To determine whether the α -thrombin Rainbow protein could be used to quantify α -thrombin-induced cleavage in a time- and dose-dependent manner, the α -thrombin Rainbow proteins GTAq and EGTAq were incubated with α -thrombin (0–13.2 units/ml). Heat inactivation of the α -thrombin prevented the change in emission ratio. α -Thrombin had no effect on GAq without a linker and also no effect with aequorin alone (results not shown). The changes in ratio were dependent on dose (3.2–13.2 units/ml) and time (Figure 6). In a similar manner to the effect of caspase-3 on EGCAq, PAGE (Figure 3) confirmed the proteolysis of EGTAq by α -thrombin.

Monitoring of GFP fluorophore formation by CRET

The emission ratios of both EGTAq and GTAq at 500 and 450 nm, measured after formation of the proteins in the wheat-germ cell-free system (see the Experimental section) were 2–3-fold greater than the emission ratio of aequorin alone at 500 and 450 nm (results not shown). However, these emission ratios were considerably smaller than those found with Rainbow proteins extracted from cells (Figures 4 and 5). This showed that some GFP formation had occurred in the transcription/translation system *in vitro*, but it was incomplete in either case. This was confirmed by a bimodal spectrum (results not shown). These results show that sufficient active Rainbow protein could be

Figure 6 Effect of α -thrombin on CRET activity of EGTAq construct: dose response and time course of α -thrombin action

The CRET chimaera EGTAq, expressed and then extracted from COS7 cells (see the Experimental section), was incubated in α -thrombin activation medium in the absence (◆) and in the presence of 3.2 (□), 6.5 (▲) and 13.2 (○) units/ml α -thrombin for up to 9 h at room temperature. Samples were taken at defined intervals and the aequorin flash emission ratios at 500 and 450 nm were estimated as described in the Experimental section.

formed for screening protease specificity and that CRET could be a useful tool for quantifying the extent of GFP fluor formation under particular conditions *in vitro* or *in vivo*.

DISCUSSION

The results reported here show that highly efficient resonance energy transfer can occur between aequorin and GFP or EGFP, when linked together by peptides at least six residues long. With no linker present, energy transfer is weak. No energy transfer was detected when the GFP and aequorin were unlinked, in free solution. The chemiluminescence spectra of GTAq and GCAq were similar that reported for GFP fluorescence, in terms of peak emission and bandwidth (Figure 1 and Table 1). A complete shift in peak emission from 460–480 nm, that of aequorin alone, to approx. 510 nm, that of GFP (Figure 1), justified our name of Rainbow proteins [25]. In the jellyfish GFP shifts the peak emission and sharpens the spectrum, decreasing the bandwidth; a similar effect was observed in all our Rainbow protein chimaeras. The slight differences in bandwidths (Table 1) were reflected in the emission ratios at 500 and 450 nm (Table 2), which is consistent with improvements in the distance or orientation of the donor and acceptor with the longer linker. We have reported previously [26] that the energy transfer efficiency of GAq chimaeras, assessed by an analysis of spectral or emission ratios, increased at least up to a linker length of 50 residues. However, insertion of a complete protein, BiP (grp78), molecular mass 78 kDa, as linker produced a ratio only 1.5-fold that of aequorin, but still significantly different from that of aequorin alone (A. Bermúdez Fajardo, unpublished work). The complete shift in spectral peak emission from aequorin to that of GFP showed that energy transfer here was superior to that reported for *Renilla* luciferase to yellow GFP [27,28]. *Renilla* luciferase is unlikely to be an ideal donor for GFP because binding of its own GFP to the luciferase occurs, even at low concentration [11].

Renilla GFP increases the quantum yield of its own luciferase approx. 3-fold, increasing it to a value similar to that of aequorin (approx. 15%). However, as expected, the apparent quantum yield for GFP-linker-aequorin was very similar to that of free aequorin. Thus GFP does not increase the quantum yield of aequorin. The differences in light emitted from cells with GFP-

linker-aequorin constructs [25] were likely to be due to better synthesis and slower proteolysis of aequorin chimaeras than of aequorin alone. Furthermore, this suggests that the quantum yield of aequorin of less than 20% is the result of energy loss in the chemi-excitation step and not in the fluorescence emission of the excited coelenteramide once it has been formed.

Any construct not containing a fully formed fluor would emit blue light and would create a bimodal spectrum. Addition of aequorin to the CRET constructs did indeed result in the appearance of a new peak due to aequorin alone. However, only a very small bimodality was observed in some experiments (see Figure 1, GTAq), suggesting that the GFP was fully formed in most constructs. A small amount of chimaera with unformed GFP fluor might also increase the bandwidth, as was seen when proteolysis of the Rainbow protein was not complete (compare Figure 2 spectra with proteolysis efficiency shown in Figure 3). The bandwidth differences between GCAq and GTAq could not be explained simply by differences in efficiency of GFP formation. It is likely that these differences were caused by differences in the orientation, rather than the distance, between donor and acceptor, because the best ratios were seen with the longest, most flexible linker (18 residues in GCAq, as opposed to six in GTAq). The efficiency of resonance energy transfer is predicted by the Förster equation (see [1,10]): energy transfer efficiency, E , equals $d^{-6}/(d^{-6} + R_0^{-6})$, where d is the distance in ångströms between the chemiluminescent (CRET) or fluorescent (FRET) donor and the fluorescent acceptor, and $R_0 = (J\kappa^2\Phi n^{-4})^{1/6} \times 9.7 \times 10^3$, where J is the spectral overlap integral between the emission spectrum of the donor and the excitation (absorption) spectrum of the acceptor; κ^2 is the orientation factor between donor and acceptor (between 0 and 4, 2/3 being random); Φ is the fluorescence quantum yield of the chemiluminescent (CRET) or fluorescent (FRET) donor; and n is the refractive index of the medium between donor and acceptor. Thus, for any significant energy transfer to take place, the donor and acceptor must be within 100 Å (10 nm). The expected separation distance between GFP and aequorin in our constructs is well within this, even when a full protein was engineered between them; we have shown previously that chemiluminescence energy transfer can occur across proteins as large as antibodies [10].

Coelenteramide, the chemiluminescent product of coelenterazine, and the emitter in aequorin [1,29,30], can exist in three ionization states, neutral (410 nm), mono-anion (470 nm) and dianion (530 nm) [18]. Only the neutral and mono-anion species would be expected to act as donors for GFP, the neutral moiety being a weak donor to any GFP-S65T variant acceptors because these have lost the 390 nm excitation peak found in wild-type GFP. Thus the proportion of these different ionization states would also be expected to affect the bandwidth of the CRET spectrum. No major differences in spectra were observed between GTAq and EGTAq, although the bandwidths of chimaeras with GFP were wider than those with EGFP. These results suggest that the 390 nm excitation peak in GFP, which is absent from GFP-S65T mutants, might not be significant as an acceptor for energy transfer. However, this needs further investigation, particularly with synthetic coelenterazine, which generates more violet emission than the wild-type emitter [29].

The substrate recognition sequence in the linkers was exposed sufficiently to be recognized by their respective proteases, both *in vitro* (Figures 2 and 3) and inside cells (Figure 4). Specificity for the linker was confirmed in that caspase-3 did not cut EGTAq, and α -thrombin did not cut EGCAq. Binding alone as the cause of changes in light emission ratio was ruled out, since both α -thrombin and caspase-3 sites were cleaved, proteolysis being confirmed by PAGE, and the extent of cleavage correlated

approximately with the extent of the change in emission ratio at 500 and 450 nm (Figures 2 and 3). Apoaequorin has been shown to have a short half-life in cells [31,32]. The results on PAGE suggest that caspase is not the proteolytic mechanism responsible for this. The effect of α -thrombin and caspase-3 were both time- and dose-dependent (Figure 6 and Table 2). Further work is required to improve the sensitivity and specificity of the Rainbow protein EGTAq for α -thrombin compared with current fluorescent substrates. However, caspase-3 Rainbow protein indicator was able to provide some interesting insights into the specificity of the active sites for caspase-3 and 9.

The caspase family are intracellular cysteine proteases that are vital in apoptosis. Group I (caspase-1, caspase-4 and caspase-5) prefer the cleavage target sequence WEHD, group II (caspase-2, caspase-3 and caspase-7) the cleavage target DEVD, and group III (caspase-6, caspase-8 and caspase-9) prefer L/VEHD [22]. However, it has been reported that these sequences might not be specific [23]. The Rainbow protein technology established here shows that it will be useful in determining both in live cells and *in vitro* the sequence specificity of such proteases. Clearly, the sequence DEVD can be recognized by both caspase-3 and caspase-9 (Figure 4 and Table 2). Both caspase-3 and caspase-9 were able to change the spectrum and emission ratio of EGCAq towards that of aequorin. However, some degree of specificity could be obtained by using the appropriate dose of an inhibitor (Figure 4). Triggering of apoptosis by staurosporine showed direct activation of caspase-3 in live cells (Figure 5). Specificity was confirmed by the addition of Z-VAD-FMK, an inhibitor of caspase activation [20], which blocked the change in emission ratio.

Precise quantification of GFP fluorophore formation, as a result of the internal cyclization of amino acids S(T)⁶⁵Y⁶⁶G⁶⁷, in live cells and *in vitro* is important if experiments using the over-expression of GFP chimaeras in cells are to be interpreted correctly. Wild-type GFP can yield a considerable amount of unfolded GFP. Although this has been improved by mutating GFP, it is still not clear exactly how much unfolded GFP is present. In the endoplasmic reticulum this is particularly important because unfolded proteins activate a stress response, through signals generated inside the endoplasmic reticulum [17,33]. Our results show that CRET can be used to quantify the rate of GFP formation. This will prove useful in determining the efficiency of GFP variants expressed in cells, providing important information when using these variants as markers for targeting or in FRET probes. Our results were consistent with faster formation of the fluorophore of EGFP than that of GFP [15].

CRET has exciting potential for monitoring chemical processes in live cells [25]. The results reported here used aequorin in photoprotein mode: data were obtained discontinuously, but important information on caspase activation in live cells was obtained. Aequorin can be engineered to work in luciferase mode [34,35], allowing CRET indicators to be used to monitor protease activity continuously in live cells. The signal-to-noise ratio in CRET is very good [10] because, unlike FRET [4,36], no excitation of the acceptor can occur except by energy transfer. Fluorescence is superior to bioluminescence in its precision for spatial resolution in individual cells. An advantage of bioluminescence is the ability to image global signals from targeted indicators, from single cells to whole organs or even organisms [3,5,26,37]. Bioluminescent probes, unlike fluors, are not susceptible to photobleaching. Rainbow proteins [25] have spectra sufficiently different from the bioluminescent donor alone to monitor multiple gene expression simultaneously, and Ca²⁺ in two subcellular compartments simultaneously, by green emission

in one compartment and blue in the other. A potential exciting application will be to use kinase and other covalent modification sites and binding proteins as linkers [25], enabling a range of signalling events to be monitored in live cells when aequorin is used in luciferase mode [34]. A wide range of other luciferases and fluors are known as possible donors and acceptors [1,12,24,38,39]. This will lead to a family of 'Rainbow proteins' that will further our understanding of intracellular signalling mechanisms, and provide a drug-screening system easily applicable to automation.

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