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REVIEW ARTICLE

Recombinant aequorin and green fluorescent protein as valuable tools in the study of cell signalling

Anna CHIESA*[1], Elena RAPIZZI*[1], Valeria TOSELLO*, Paolo PINTON†, Maddalena DE VIRGILIO*, Kevin E. FOGARTY‡ and Rosario RIZZUTO*[2]

*University of Ferrara, Department of Experimental and Diagnostic Medicine, Section of General Pathology, Via L. Borsari 46, 44100 Ferrara, Italy, †University of Padua, Department of Biomedical Sciences, Via G. Colombo 3, 35121 Padua, Italy, and ‡Biomedical Imaging Group, University of Massachusetts Medical School, 373 Plantation St., Worcester, MA 01655, U.S.A.

Luminous proteins include primary light producers, such as aequorin, and secondary photoproteins that in some organisms red-shift light emission for better penetration in space. When expressed in heterologous systems, both types of proteins may act as versatile reporters capable of monitoring phenomena as diverse as calcium homoeostasis, protein sorting, gene expression, and so on. The Ca^{2+}-sensitive photoprotein aequorin was targeted to defined intracellular locations (organelles, such as mitochondria, endoplasmic reticulum, sarcoplasmic reticulum, Golgi apparatus and nucleus, and cytoplasmic regions, such as the bulk cytosol and the subplasmalemmal rim), and was used to analyse Ca^{2+} homoeostasis at the subcellular level. We will discuss this application, reviewing its advantages and disadvantages and the experimental procedure. The applications of green fluorescent protein (GFP) are even broader. Indeed, the ability to molecularly engineer and recombinantly express a strongly fluorescent probe has provided a powerful tool for investigating a wide variety of biological events in live cells (e.g. tracking of endogenous proteins, labelling of intracellular structures, analysing promoter activity etc.). More recently, the demonstration that, using appropriate mutants and/or fusion proteins, GFP fluorescence can become sensitive to physiological parameters or activities (ion concentration, protease activity, etc.) has further expanded its applications and made GFP the favourite probe of cell biologists. We will here present two applications in the field of cell signalling, i.e. the use of GFP chimaeras for studying the recruitment of protein kinase C isoforms and the activity of intracellular proteases.

Key words: calcium homoeostasis, chemiluminescence, fluorescence, protein kinase C, signal transduction.

INTRODUCTION

In the past two decades, our understanding of how extracellular signals are conveyed to eukaryotic cells via an increase in the intracellular Ca^{2+} concentration has expanded greatly. It is now common knowledge that a variety of extracellular stimuli (ranging from the binding of hormones, neurotransmitters and growth factors to phenomena such as cell–cell interactions), through diverse mechanisms (e.g. receptors that are themselves ion channels, or have an intrinsic enzymic activity or are coupled to enzymic effectors via G-proteins), induce a rise in the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$) of defined amplitude and kinetics [1,2]. Moreover, the technological advancements in probe design and imaging systems, by allowing the accurate measurement of Ca^{2+} concentration at the single-cell level, have revealed a marked asynchronicity in cell response and high spatio–temporal complexity of the intracellular Ca^{2+} signal. We now know that the Ca^{2+} signal can be conveyed as repetitive $[Ca^{2+}]_c$ spikes (commonly referred to as Ca^{2+} oscillations) [3,4] as well as localized increases in $[Ca^{2+}]_c$ that may either be confined or propagate gradually to the rest of the cell ('Ca^{2+} waves') [5,6]. Pivotal in unravelling such a complex scenario has been the availability of specific and useful probes, such as the fluorescent dyes developed by R. Y. Tsien and co-workers [7,8]. These probes are non-traumatically loaded into living cells by adding the ester form to the incubation medium: the esters permeate across the cell membrane and are trapped inside the cell by the action of cell esterases, which restore the Ca^{2+}-binding form of the indicator and trap it in the cell. This simplicity of use, combined with the intense fluorescence, has allowed the detailed investigation of Ca^{2+} signalling, in populations and single cells, in virtually every cell type, revealing the great complexity described above.

The interesting challenge currently is understanding how the complexity is generated and how it is decoded by the cell. For this task, fluorescent indicators are not sufficient. Indeed, in order to study the changes in Ca^{2+} concentration that occur in defined cell compartments (organelles, cytosolic subregions, the microenvironment of Ca^{2+}-sensitive proteins, etc.), Ca^{2+} probes with highly selective intracellular locations are needed. In the first part of this article, we will describe the construction strategy, the mode of employment and a couple of examples of a novel class of Ca^{2+} probes, intracellularly targeted chimaeras of the Ca^{2+}-sensitive photoprotein aequorin. In the elucidation of the complexity of Ca^{2+} signalling, analysis of the downstream effects is a logical follow-up. In the second part of the article, we will describe a microscopy approach for monitoring the recruitment of the various isoforms of a classical Ca^{2+} effector, protein kinase C (PKC) [9]. For this purpose, we employ chimaeras of green fluorescent protein (GFP) with different spectral properties

Abbreviations used: BFP, blue flourescent protein; BRET, bioluminescence resonance energy transfer; $[Ca^{2+}]_c$, cytoplasmic Ca^{2+} concentration; ER, endoplasmic reticulum; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; eGFP (etc.), 'humanized' mutant of wild-type GFP (etc.); HA1, short epitope tag derived from haemagglutinin; KRB, Krebs–Ringer buffer; PKC, protein kinase C.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed (e-mail rzr@dns.unife.it).

[10] and a high-resolution imaging system, based on the computational deblurring of wide-field fluorescence images.

AEQUORIN

General properties of aequorin

Aequorin is a Ca^{2+}-sensitive photoprotein derived from a marine organism, the jellyfish *Aequorea victoria* [11,12]. The structure of aequorin has recently been solved, providing further insight into the molecular mechanism of chemiluminescence [13]. Aequorin is composed of an apoprotein (molecular mass ~ 21 kDa) and a hydrophobic prosthetic group, coelenterazine (molecular mass ~ 400 Da), and its polypeptide sequence includes three high-affinity Ca^{2+}-binding sites. Ca^{2+} binding causes the rupture of the covalent link between the apoprotein and the prosthetic group, a reaction associated with the emission of one photon (Figure 1a). The rate of this reaction depends on the Ca^{2+} concentration to which the photoprotein is exposed. Indeed, at saturating Ca^{2+} concentrations ($> 100\ \mu M$) the reaction is virtually instantaneous and the complete photoprotein pool is discharged. At lower Ca^{2+} concentrations, there is a relationship between the fractional rate of consumption and Ca^{2+} concentration, which has been characterized extensively for the cellular milieu (temperature, ionic conditions, etc.) (Figure 1b) [14,15]. Thus, despite the irreversible nature of the aequorin reaction (as distinct from fluorescent Ca^{2+} dyes, only one photon can be collected from one aequorin molecule during an experiment), the Ca^{2+} concentration of a sample can be calculated from luminescence data in prolonged (tens of minutes) experiments. Based on these properties, aequorin has been used extensively as a Ca^{2+} probe for the cytoplasm of intact living cells, allowing seminal observations to be made (such as, for example, the occurrence of repetitive spiking of $[Ca^{2+}]_c$ upon agonist stimulation) [16]. In these experiments, purified aequorin, extracted from the jellyfish, was microinjected into living cells, as it cannot penetrate the membrane of intact cells. This has limited the number of applications and cell types that could be employed, and indeed aequorin has been largely replaced by the fluorescent dyes as the preferred probe for measuring $[Ca^{2+}]_c$ in living cells.

The cloning of the aequorin cDNA [17] has opened up new perspectives to the use of this Ca^{2+} probe, for two main reasons. The first is that recombinant expression circumvents the need for microinjection, or other traumatic and/or laborious techniques for introducing the polypeptide into living cells. Indeed, in the experience of our and numerous other laboratories, the cDNA can be successfully transfected and expressed in a wide variety of cell types (mammalian cells, plants, yeasts, etc.), thus allowing the production of the recombinant apoprotein [18–20]. As to the formation of the holoprotein, this is achieved by adding exogenously the prosthetic group (which permeates freely across cell membranes), and allowing sufficient time for its post-translational incorporation (a procedure, named reconstitution, that will be discussed later). The second, more important, reason for the renewed interest in aequorin is that, being a protein, it can be molecularly modified by adding specific targeting sequences. Thus protein chimaeras can be constructed which are endowed with a defined intracellular localization. By this approach, as we will discuss later, aequorin can be targeted to a variety of organelles and cell compartments, thus obtaining novel tools for investigating the spatio–temporal complexity of calcium signalling. In this part of the article, we will briefly review the principles of aequorin utilization, and give some representative examples.

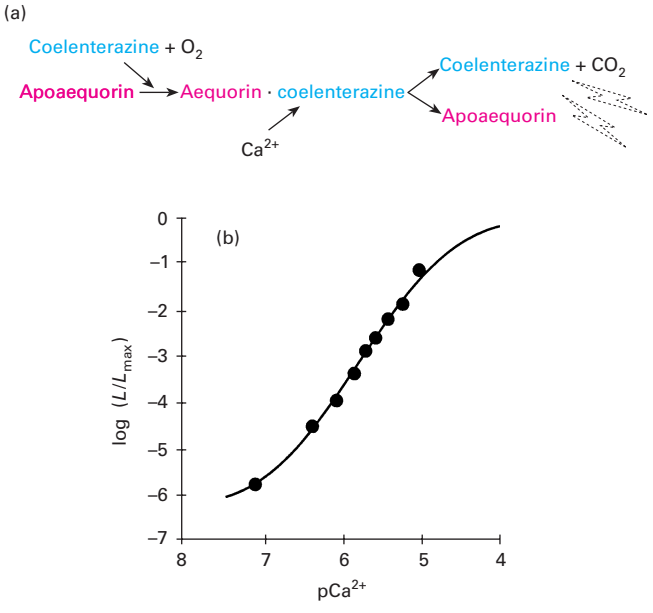

Figure 1 (A) The aequorin reaction, and (B) the Ca^{2+} concentration response curve of recombinant aequorin

The fractional rate of aequorin consumption is expressed as the ratio between the emission of light at a defined Ca^{2+} concentration (L) and the maximal rate of light emission at a saturating Ca^{2+} concentration (L_{max}).

Modifying the aequorin cDNA

Introducing an epitope tag

As discussed, the crucial advantage of recombinant aequorin is its selective intracellular localization. Given that most aequorin measurements are carried out in populations of cells (and thus the localization cannot be derived by the direct analysis of aequorin luminescence), it is essential to demonstrate that the recombinant photoprotein is sorted to the expected cell location. This is achieved by immunocytochemical labelling of transfected cells. Given that aequorin is itself a fairly poor immunogen (and thus we do not have very good antibodies available), we have appended a short (9-amino-acid) epitope tag (HA1, derived from haemagglutinin) to its protein sequence [21]. This HA1-tagged aequorin has been employed as the 'building block' for all the chimaeras described here.

Modifying the affinity of the photoprotein

Although aequorin is endowed with a wide dynamic range (thus allowing the monitoring of increases in Ca^{2+} concentration ranging from $0.5\ \mu M$ to approx. 5–$10\ \mu M$), the high rate of consumption in the cell compartments endowed with higher $[Ca^{2+}]_c$ (typically the intracellular Ca^{2+} stores) impairs the use of aequorin as a reliable Ca^{2+} probe in these domains. For this purpose, it is necessary to lower the affinity of the photoprotein. This can be achieved by three different approaches, which can be combined to obtain a Ca^{2+} probe with very low affinity. The first is mutation of the Ca^{2+}-binding sites. Indeed, all our chimaeras destined for compartments with high Ca^{2+} concentrations include a mutation (Asp-119 → Ala), described by Kendall and co-workers [22], that causes an approx. 20-fold decrease in the Ca^{2+} affinity of the photoprotein. The second approach is based on the use of a modified prosthetic group (coelenterazine n) that further decreases the affinity of the mutated photoprotein [23]. In our

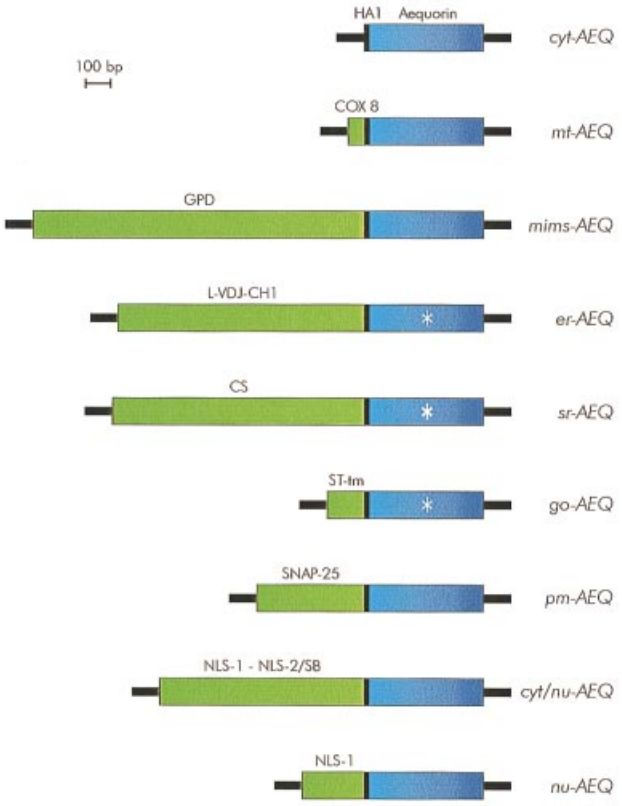

Figure 2 Schematic representation of aequorin chimaeras targeted to specific subcellular locations

The chimaeras represented localize to the cytoplasm (*cyt-AEQ*), the mitochondrial matrix (*mt-AEQ*), the mitochondrial intermembrane space (*mims-AEQ*), the ER (*er-AEQ*), the sarcoplasmic reticulum (*sr-AEQ*), the Golgi apparatus (*go-AEQ*), the subplasmalemma region (*pm-AEQ*), the cytosol or nucleus (depending on presence of glucocorticoids; *cyt/nu-AEQ*) or the nucleus only (*nu-AEQ*). A white asterisk within the aequorin portion denotes the D119A mutation. Abbreviations are as follows: COX 8, N-terminal fragment of subunit VIII of cytochrome *c* oxidase; GPD, glycerol phosphate dehydrogenase; L-VDJ-CH1, domains of an Igγ2b heavy chain; CS, calsequestrin; ST-tm, sialyltransferase transmembrane domain; SNAP-25, synaptosomal-associated protein of 25 kDa; NLS, nuclear localization signal; SB, steroid-binding domain.

experience, the use of the low-affinity aequorin mutant reconstituted with coelenterazine n allows accurate measurements of Ca^{2+} concentration in the Ca^{2+} stores, and thus no further procedure needs to be employed. In any case, it should be remembered that a drastic decrease in aequorin light emission (and thus probe consumption) can be obtained by a third approach, the substitution of Ca^{2+} in the live sample with the surrogate ion Sr^{2+} [24].

Targeting aequorin to intracellular organelles

Targeting, and use, of aequorin has proven successful in a wide variety of cell locations. A partial representative list of cDNA constructs, summarizing the targeting strategies employed, is presented in Figure 2. For the cytoplasm, no further modification is introduced in HA1-tagged aequorin. For the mitochondrial matrix, HA1-tagged aequorin is fused to the cleavable targeting sequence (mitochondrial presequence) of a subunit (VIII) of cytochrome *c* oxidase, the terminal complex of the respiratory chain [18]. The recombinant chimaera is localized in the mitochondria, being freely diffusible in the matrix (as assessed by immunocytochemical and biochemical tests). For the mitochondrial intermembrane space, aequorin is fused to the C-terminus of glycerol phosphate dehydrogenase, an integral protein of the mitochondrial inner membrane that has a large C-terminal domain protruding into the intermembrane space [25]. The sorting to mitochondria has been verified by immunocytochemistry, and the correct topological orientation of aequorin can be inferred unambiguously by properties of the Ca^{2+} signals detected by the probe. For the nucleus, two types of probe have been generated. A constitutively nuclear probe is obtained by fusing aequorin to a small portion (amino acids 407–524, including the nuclear localization signal) of the glucocorticoid receptor [26]. A similar approach has been employed successfully by Campbell and co-workers [27]. In order, however, to detect minor differences between the nucleus and the cytoplasm, a 'shuttling' probe was developed, which can be directed to either compartment based on the experimental conditions. This chimaera is composed of aequorin and a larger portion of the glucocorticoid receptor, thus encompassing the hormone-binding site and allowing hormone-dependent translocation to the nucleus. This probe is therefore cytoplasmic in the absence of glucocorticoid hormones, and nuclear in their presence [28]. To target apoaequorin to the endoplasmic reticulum (ER), two approaches have been developed. In the first, Kendall and co-workers fused a hydrophobic ER insertion signal derived from calreticulin and appended at the N-terminus of aequorin, and a KDEL sequence appended at the C-terminus [29]. In our

laboratory, we have fused aequorin–HA1 to the N-terminal region of the immunoglobulin heavy chain, which includes the leader sequence (L), the VDJ and the CH1 domains of an IgG heavy chain [24]. Via the L and CH1 domains respectively, both insertion in the ER and retrieval from later compartments are achieved, thus allowing the exclusive sorting of the chimaera to the ER without modifying the C-terminus, a procedure that has been reported to affect aequorin luminescence drastically [30]. With regard to the sarcoplasmic reticulum and the Golgi apparatus, targeting of aequorin is achieved via fusion to two resident proteins, calsequestrin and sialyltransferase respectively [31,32]. Finally, aequorin is directed to the subplasmalemmal rim of the cytoplasm by fusing it to SNAP-25 (25 kDa synaptosome-associated protein), a protein that is synthesized in the cytoplasm and is translocated to the plasma membrane upon post-translational palmitoylation of cysteine residues [33]. We had previously constructed a fusion protein with a plasma membrane receptor, but in that case targeting to the plasma membrane was quite inefficient (the chimaera was largely retained in intracellular vesicles).

Expressing and reconstituting the functional protein

In transient expression experiments, cells are usually transfected by the calcium phosphate method, which in most cases proves to be the simplest and most reproducible procedure. For each aequorin measurement, the cells are seeded on to a 13 mm coverslip and allowed to grow to 50 % confluence. At this stage, cells are transfected with the appropriate plasmid 4 μg/coverslip), and then analysed for aequorin expression after 36 h. This procedure is employed successfully in a variety of cell models, including HeLa, L929, L cells, Cos7 and primary cultures of cortical neurons, colangiocytes and skeletal myotubes. For the last of these, the procedure is slightly modified, as transfection is carried out on the second day of culture (i.e. at the stage of myoblasts) and the cells are analysed on day 8, i.e. when optimal expression in fused myotubes is achieved [31]. Other transfection procedures can be employed for cell types in which calcium phosphate proves to be quite ineffective, e.g. we use electroporation with GH3 and Ins1 cells, and PEI (polyethyleneimine) with Jurkat cells.

As mentioned above, recombinantly expressed aequorin is only the polypeptide portion of the photoprotein. In order to measure changes in Ca^{2+} concentration, it is necessary to reconstitute it in the active form. To this end the prosthetic group coelenterazine (dissolved at a final concentration of 0.5 mM in pure methanol and kept in aliquots at -80 °C in the dark) is added to the transfected cells using the following procedure.

In order to reconstitute targeted aequorin expressed in cell compartments with low Ca^{2+} concentrations, such as the cytosol, nucleus and mitochondria, the coverslip with the transfected cells is incubated with 5 μM coelenterazine for 2 h in Dulbecco's modified Eagle's medium supplemented with 1 % (v/v) fetal calf serum at 37 °C in a 5 % CO_2 atmosphere, and then transferred directly to the luminometer chamber, where the incubation buffer is switched to modified Krebs–Ringer buffer (KRB, containing 125 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, 5.5 mM glucose and 20 mM Hepes, pH 7.4, at 37 °C), supplemented with 1 mM $CaCl_2$.

In the case of cell compartments with high Ca^{2+} concentrations (e.g. ER, sarcoplasmic reticulum and Golgi apparatus), where the high rate of aequorin consumption would strongly counteract the reconstitution process, it is necessary to drastically lower the lumenal Ca^{2+} concentration before adding the prosthetic group. To this end, the cells are incubated for 1 h at 4 °C in KRB supplemented with coelenterazine (5 μM), the Ca^{2+} ionophore ionomycin (5 μM) and 600 μM EGTA. After this incubation, cells are washed extensively with KRB supplemented with 2 % (w/v) BSA and 1 mM EGTA before being transferred to the luminometer chamber.

The reconstitution of the aequorin chimaera targeted to the plasma membrane is decreased drastically in media containing physiological Ca^{2+} concentrations (1 mM). In order to increase the amount of active aequorin, the transfected cells are transferred to modified KRB supplemented with 100 μM EGTA and 5 μM coelenterazine. After 45 min of incubation at 37 °C, the coverslip is transferred to the luminometer chamber.

Recording and calibrating the aequorin signal

The feeble light emission of aequorin (and of photoproteins in general) renders direct imaging at the single-cell level a quite difficult task, which requires sophisticated imaging apparatus and is endowed with relatively low resolution. Rutter and colleagues [34], by increasing the expression of the protein through intranuclear injection of cDNA and using a detector based on a dual microchannel plate intensifier, succeeded in imaging Ca^{2+} concentration with a recombinant chimaeric aequorin. For imaging studies, enhancing aequorin light emission would be highly beneficial. In this respect, an interesting perspective is provided by the recent demonstration that energy transfer between aequorin and its natural fluorescent partner (GFP) may occur in chimaeras including the two proteins, and improves the quantum yield of the bioluminescent process [35].

On the other hand, the excellent subcellular distribution of the recombinant probes allows us to obtain much information from population studies, which are therefore the typical experiment carried out in the laboratory. After transfection with the appropriate aequorin cDNA, the cells are transferred to the perfusion chamber of a purpose-built luminometer [36]. While we refer to the original article for a detailed description, we will briefly summarize here the principles of the instrumentation, represented schematically in Figure 3. The perfusion chamber resides in a notch (diameter 15 mm, height 2 mm) at the top of a hollow cylinder filled with water thermostatted at 37 °C. After fitting the coverslip, the chamber, sealed on top with a larger coverslip, is placed, in a dark box, in close proximity to a low-noise photomultiplier, with a built-in amplifier/discriminator. The pulses generated by the discriminator are captured by the photon-counting board of an IBM-compatible computer and stored, in order to carry out the off-line calibration of the aequorin signal into Ca^{2+} concentration values. While maintained in a dark box, the chamber with the cells is perfused continuously, via plastic tubing, with KRB (pH 7.4 at 37 °C) containing 1 mM $CaCl_2$. During a typical experiment, all additions to the cells are made by supplementing the perfusion medium with the compound of interest (agonists, inhibitors, etc.). During the change of medium, the rate of perfusion is raised from 2.5 to 5 ml/min, in order to allow rapid equilibration of the compounds in the perfusion chamber. At the end of each experiment, the cells are lysed by the perfusion of a hypotonic solution containing digitonin (100 μM) and $CaCl_2$ (10 mM). In this way, aequorin that was not consumed during the experiment is discharged, and it is possible, at this stage, to estimate the total aequorin content (L_{max}) of the sample. The luminescence data are then processed by an algorithm that back-calculates, throughout the experiment, L/L_{max}, where L is the light emission at a given point and L_{max} is the maximal light emission (a value that decreases gradually with the consumption of the probe). Given that, for physiological conditions of pH, Mg^{2+} concentration, temperature and ionic strength, the re-

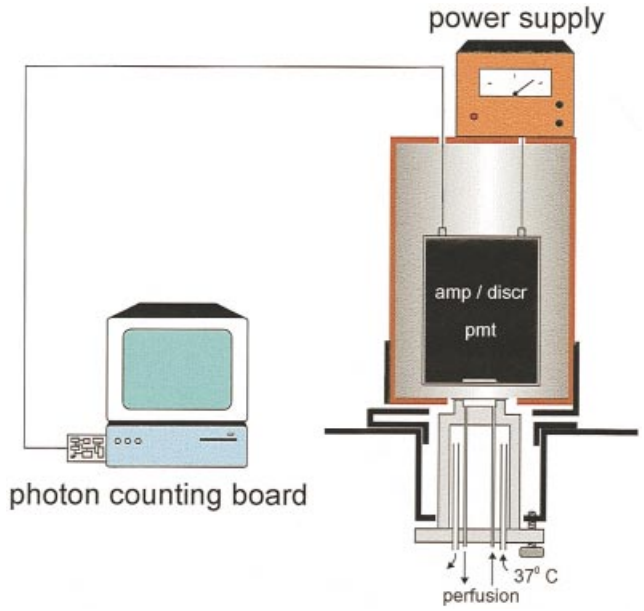

Figure 3 Schematic representation of a custom-built luminometer

Cells expressing functional aequorin probe are incubated in a perfusion chamber, at 37 °C, in close proximity to a photon-counting tube. The complete assemblage is in the dark, to minimize extraneous signals. The luminescence data are acquired by an IBM-compatible computer via a photon-counting board, and conversion of light signal into Ca^{2+} concentration is carried out using an algorithm based on the Ca^{2+} response curve of aequorin. Abbreviations: amp/discr, amplifier/discriminator; pmt, photomultiplier tube.

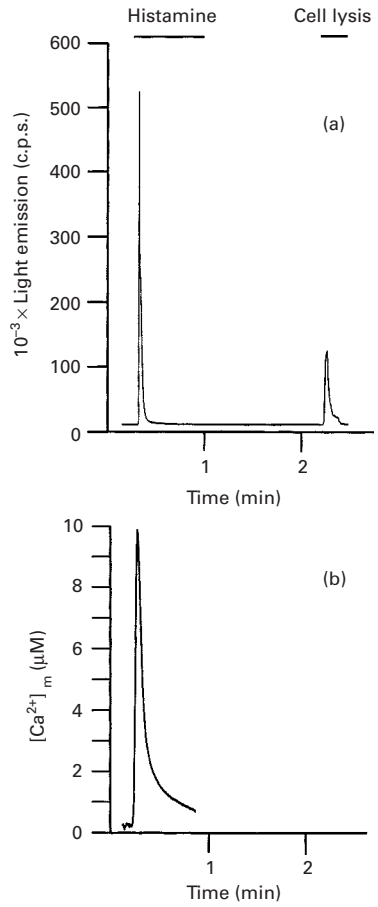

Figure 4 Calibration of light data into Ca^{2+} concentration values

Shown are light emission (**a**) and calculated values for the mitochondrial Ca^{2+} concentration $[Ca^{2+}]_m$ (**b**) from a monolayer of HeLa cells expressing mitochondrial aequorin. Where indicated, the cells were challenged with 100 μM histamine. At the end of the experiments the cells were lysed with 100 μM digitonin in a hypotonic Ca^{2+}-rich solution (10 mM $CaCl_2$ in water) to estimate the total photoprotein pool.

lationship between L/L_{max} and Ca^{2+} concentration is known, the algorithm can then calculate the Ca^{2+} concentration throughout the experiment. The same calculations can be carried out with the mutated aequorins and/or modified prosthetic groups, via algorithms based on the calcium response curves of these modified aequorins [23].

An example of the calibration of the light emission data into Ca^{2+} concentration values is reported in Figure 4. In this case, HeLa cells were transfected with plasmids expressing mitochondrial aequorin, and analysed 36 h after transfection. Aequorin light emission, and the calculated Ca^{2+} concentration data, are shown in Figures 4(a) and 4(b) respectively. It is apparent that the steepness of the response curve of aequorin endows the photoprotein with an excellent signal-to-noise ratio: 10–20 c.p.s. for resting Ca^{2+} concentration values (100–200 nM), increasing to $> 5\times10^5$ c.p.s. at the peak of agonist stimulation ($\sim 10\ \mu$M).

In Figure 5, the measurement of agonist-dependent changes in Ca^{2+} concentration in three different intracellular compartments (cytoplasm, mitochondria and ER, each monitored, in parallel batches of cells, with the appropriate aequorin chimaera) is shown. Figure 5(a) shows the data with cytoplasmic aequorin. At rest, a basal value of approx. 0.2 μM can be detected (it should be remembered, however, that, due to the steepness of the calcium response curve, aequorin estimates tend to be inaccurate for values below 0.2–0.3 μM). Upon stimulation with histamine, an agonist coupled, via G_q proteins, to the generation of inositol 1,4,5-trisphosphate and thus the release of Ca^{2+} from intracellular stores, a rise in $[Ca^{2+}]_c$ can be detected. This increase has biphasic kinetics: a rapid, transient peak ($2.5\pm0.3\ \mu$M), mostly due to the release of stored Ca^{2+}, and a more sustained plateau, due to the entry of Ca^{2+} through the plasma membrane channels activated by the depletion of internal stores. Upon histamine washout, $[Ca^{2+}]_c$ returns to basal values. Interestingly, this quite classical cytoplasmic response closely mimics, in both kinetics and absolute values, the results obtained with fluorescent dyes (not shown), thus suggesting that no significant artefact is introduced by the calibration procedure of either of these radically different probes. Figure 5(b) reports a representative trace of Ca^{2+} concentration measurements in cells expressing mitochondrial aequorin. In this case, stimulation with histamine causes a rise in the mitochondrial Ca^{2+} concentration that greatly exceeds the cytoplasmic increase (peak value $\sim 10\ \mu$M), an observation that reversed the commonly held concept that mitochondria do not accumulate Ca^{2+} significantly in healthy cells (and prompted a remarkable renaissance of these organelles in the field of calcium signalling; see Rizzuto et al. [37] for a review). Interestingly, recent work by Alvarez and co-workers [38] using a low-affinity variant of mitochondrial aequorin indicated that, at least in some cell types, the agonist-dependent Ca^{2+} concentration *in vivo* may reach values as high as 500 μM. Finally, Figure 5(c) shows the measurement of ER Ca^{2+} concentration. As discussed previously, aequorin reconstitution is carried out after greatly decreasing the Ca^{2+} concentration, thus preventing consumption of the probe

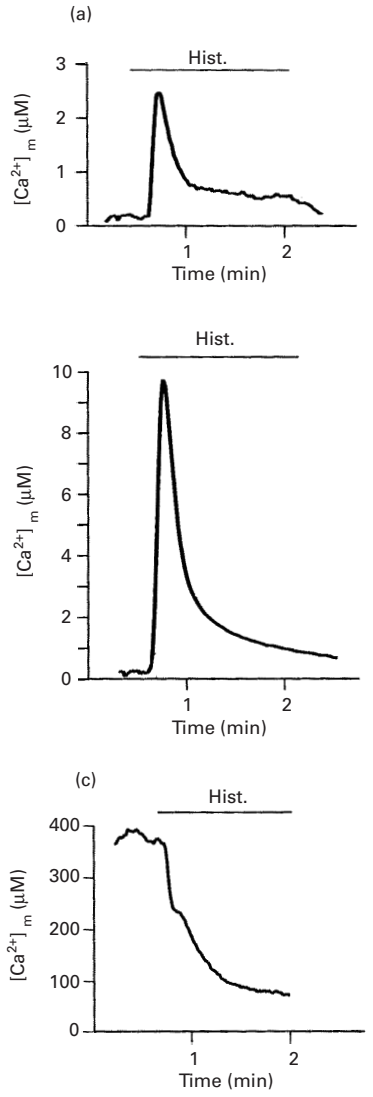

Figure 5 Measurement of Ca^{2+} concentration using cytosolic (a), mitochondrial (b) and ER (c) aequorin probes

Where indicated, the cells were challenged with 100 μM histamine (Hist.) (see the text).

during the reconstitution process and allowing the start of the experiment with a sizeable pool of active aequorin ($> 10^7$ c.p.s.). After transferring the coverslip to the luminometer chamber, the cells were perfused with Ca^{2+}-free saline solution (KRB supplemented with 100 μM EGTA). $CaCl_2$ (1 mM) was re-added where indicated, thus initiating the re-filling of the intracellular Ca^{2+} stores. At equilibrium, a steady-state luminal Ca^{2+} concentration of ~ 450 μM was reached. Stimulation with histamine caused a large, rapid fall in the ER Ca^{2+} concentration (down to a value of approx. 100 μM). Upon agonist wash-out, Ca^{2+} was re-accumulated in the ER, reaching pre-stimulatory values (results not shown).

GFP

GFP is in Nature the legitimate partner of aequorin. Indeed, it is produced by the same jellyfish (*Aequorea victoria*) and is packed in close association with the photoprotein, acting as a natural fluorophore that absorbs the blue light emitted by the photoprotein and re-emits photons of a longer wavelength (thus accounting for its own name and for the greenish hue of the jellyfish luminescence) [11,39,40]. In research applications, GFP retains its fluorescence properties, and thus can be added to the long list of probes of the cell biologist's toolbox [10]. Some of its unique properties account for its explosive success, and has made it, in relatively few years (the first report of GFP expression in heterologous systems dates back to 1994) [41], a powerful and versatile tool for investigating virtually all fields of cell biology (ranging from the study of gene expression to protein sorting, organelle structure, measurement of physiological parameters in living cells, etc.) [42,43].

The main reason for the success of GFP is its own nature: the fluorescent moiety is a gene product (with no need for cofactors) that is open to molecular engineering and transient or stable expression in virtually every cell type. Moreover, its mutagenesis has allowed the adaptation of its fluorescent properties to different experimental needs (for a detailed description, see reviews [10,44]). GFP mutants can be grouped into two major classes. The first are the 'optimizing' mutations, i.e. those that increase light emission by either altering the intrinsic properties of the fluorescent protein [45,46] or increasing its production in mammalian cells [47]. The second group are the so-called 'humanized' versions of the cDNA, in which silent mutations are introduced that convert some of the codons into the most common and efficient for translation in mammalian cells. Thus, for equal amounts of mRNA, more fluorescent protein is produced. This is very useful at low mRNA levels (e.g. for the prompt detection of promoter activity). As to the modification of GFP properties, mutations have been described that alter the stability of the protein and/or the quantum efficiency upon illumination with visible light (native GFP has a bimodal excitation peak, larger with UV than with blue light, while all currently employed green variants of GFP are best excited with blue light, thus minimizing cytotoxicity and photobleaching). Among these mutations, the most useful appears to be the substitution of Ser-65 by Thr (S65T), which causes the chromophore to be entirely in the anionic form; thus, when compared with wild-type GFP, the quantum efficiency upon excitation with blue light is increased 6-fold, the rate of fluorophore formation is 4-fold faster and photobleaching is markedly reduced. The second class is represented by GFPs emitting light of a wavelength that can be clearly distinguished from the green colour of native GFP. This class includes some popular mutants commonly referred to as blue (Y66H/Y145F), cyan (Y66W) and yellow (T203Y) GFPs.

The search, by mutagenesis, for a truly 'red' GFP mutant has been extensive, but unsuccessful [48]. Recently, however, a red companion of GFP, RFP, has been isolated from a coral, and is currently utilized in numerous laboratories [49]. Finally, while reviewing the properties of GFP, it should be remembered that, with the notable exception of the blue mutant, GFP is strongly resistant to photobleaching (thus allowing prolonged visualization under the laser illumination of confocal microscopes). For this reason, GFP has often replaced in various applications probes that were already available.

Another GFP, found in the sea pansy *Renilla*, has been characterized biochemically [50], but the cDNA has not yet been isolated. Although the chromophore of *Renilla* is similar to that of *Aequorea*, some of their biochemical properties differ. *Renilla* GFP has a much higher molar absorption coefficient, is an obligate dimer and is more resistant to pH-induced conformational changes, which could make it useful for some cell physiology studies.

Nowadays the most frequent application of the GFPs is to use them as a tag. In fact, by fusing in-frame the GFP cDNA to a cDNA coding for a protein of interest, it is possible to examine the function and fate of the resulting chimaera in living cells. Moreover, chimaeras with different spectral properties can be employed for visualizing simultaneously two proteins of interest (e.g. two isoforms of a signalling molecule) or the morphology and spatial relationship between two intracellular compartments, such as ER and mitochondria [25].

GFPs can also be used as a tool for analysing transfection efficiency. In 1994 Chalfie and co-workers [41] demonstrated the possibility of using GFPs to monitor gene expression in prokaryotic (*Escherichia coli*) and eukaryotic (*Caenorhabditis elegans*) living cells. However, some of the properties of GFP (long life-time, time required for fluorophore formation, background fluorescence) are not ideally suited for the analysis of promoter activity. Indeed, Rutter and co-workers [51] imaged gene transcription at the single-cell level using both GFP and luciferase, and showed that the latter appears preferable, given that GFP analyses are hampered by the lack of linearity between protein concentration and fluorescence intensity, as well as the interference of cellular autofluorescence.

Furthermore, GFPs can be used as sensors for physiological parameters. This approach stems from an interesting phenomenon, called fluorescence resonance energy transfer (FRET), that occurs between two GFPs of different colours. FRET may occur only if the fluorescence emission spectrum of the 'donor' GFP overlaps with the excitation spectrum of the 'acceptor' GFP and if the two fluorophores are located within few nanometres in a favourable orientation. Any alterations of these parameters can drastically alter the efficiency of FRET. Indeed, the rate of energy transfer (E) is proportional to the sixth power of the distance {i.e. $E = [1 + (R/R_0)^6]^{-1}$, where R and R_0 are the actual distances between the chromophores and the distance at which FRET is 50 % efficient respectively}, and thus becomes negligible when the two fluorophores are $>$ 5–6 nm apart. The earliest use of FRET between GFP mutants was reported by Heim and Tsien [52], who linked two fluorophores via a sequence of 25 amino acids, including a trypsin cleavage site. After addition of trypsin, the short peptide was cleaved and FRET was drastically reduced. Mitra et al. [53] used a similar approach for the substrate for factor Xa. Another use of FRET to monitor protease activity was described by Xu et al. [54], who monitored the decrease of FRET in apoptotic cells transiently expressing a chimaera between blue fluorescent protein (BFP) and eGFP (a 'humanized' mutant of wild-type GFP) linked together by a sequence including the DEVD (Asp-Glu-Val-Asp) recognition site for caspase 3. A similar approach was employed by Mahajan and co-workers [55], who linked BFP with GFP through a four-amino-acid sequence, Tyr-Val-Ala-Asp, that is specifically cleaved by caspase 1, and through a DEVD linker for caspase 3. FRET has also been used by different groups to monitor changes in intracellular Ca^{2+} concentrations. Romoser and co-workers [56] fused BFP and GFP via a linker that included a calmodulin-binding site derived from smooth muscle myosin light chain kinase. Addition of Ca^{2+}/calmodulin to this construct disrupted FRET, probably influencing the correct orientation of the fluorophores. Miyawaki et al. [57] developed a chimaera called CaMeleon, in which appropriate GFP variants were linked by a peptide that included the N-terminal part of calmodulin and the M13 peptide that associates with calmodulin in a Ca^{2+}-dependent manner. Moreover, by targeting this chimaera to the ER, the authors were able to measure the lumenal Ca^{2+} concentration of this organelle. Finally, FRET between GFP mutants can be employed for monitoring protein–protein interactions, such as, for example, those between Bcl2 and Bax [58] and between epidermal growth factor receptor and Grb2 [59]. A new biophysical approach, known as bioluminescence resonance energy transfer (BRET), has recently been developed. BRET is a phenomenon resulting from radiation-less energy transfer between a luminescent donor (i.e. with no need of external illumination) and fluorescent acceptor proteins. Using this approach, and taking advantage of emission spectral overlap between the bioluminescent *Renilla* luciferase and yellow fluorescent protein, Xu and colleagues [60] demonstrated homodimerization of the cyanobacteria clock protein KaiB in *Escherichia coli*. Along the same lines, Angers et al. [61] used BRET to reveal dimerization of β_2-adrenergic receptors in HEK-293 living cells.

Experimental set-up: collecting and analysing the GFP images

The fluorophore of GFP is formed by the cyclization of three amino acid residues of the primary sequence. This process directly follows, with few constraints (a relatively brief time lag), the synthesis of the protein, and thus GFP proves to be brightly fluorescent when expressed in a wide variety of cell types (mammalian cells, plants, fungi, bacteria, etc.) and intracellular locations (cytoplasm and virtually every organelle). Thus in our experiments the various GFP chimaeras are transfected using the appropriate procedure (calcium phosphate, liposomes or particle gun, depending on the cell type), and visualized directly in living cells after allowing sufficient time for expression and chromophore formation (usually 24–36 h). For this purpose, the coverslip with the transfected cells is fitted at the base of a thermostatted chamber, which is placed on the microscope stage.

Our microscope set-up is presented in Figure 6. In brief, a traditional wide-field, epifluorescence microscope is equipped with filter-wheels located both in the incoming and in the outcoming light paths (thus allowing rapid alternation of excitation and/or emission wavelength) and a piezoelectric transducer (or motor drive) for rapid focusing in the z plane. The fluorescence image is collected by a back-illuminated and cooled (-40 °C) charge-coupled device (CCD) camera having high quantum efficiency (70 % at 500 nm) and low noise ($< 10\ e^-$ root mean square at 1 MHz) characteristics, and the image is stored as a digital file using the Metamorph/Metafluor program (Universal Imaging). This allows the direct monitoring of fluorescence intensity, which is important for some applications (e.g. the monitoring of FRET, or pH measurements using pH-sensitive GFP mutants, a topic that for reasons of brevity will not be discussed in this article).

A high-resolution, three-dimensional reconstruction of the distribution of a GFP chimaera can be obtained by the technique of digital image restoration, also called deconvolution or deblurring. While we refer to more exhaustive descriptions of this approach [62], a clear demonstration of the improvement of the image quality can be obtained from the example in Figure 8 below. A series of 20 optical section images, spaced at 0.5 μm intervals through the depth of the cell, was acquired. Each optical section image was acquired in less than 1 s, and the entire through-focus series in less than 20 s. The image series was then deblurred using a constrained, iterative restoration algorithm that incorporates an empirically determined optical point spread function. The deblurred images were then used to visualize the three-dimensional intracellular patterns.

In situations where there may be significant intracellular motion of the GFP chimaera, two approaches can be used to decrease the time required for image acquisition. The area of the cell (i.e. the number of pixels in each optical section) imaged can be reduced, thus decreasing the time taken to transfer the image

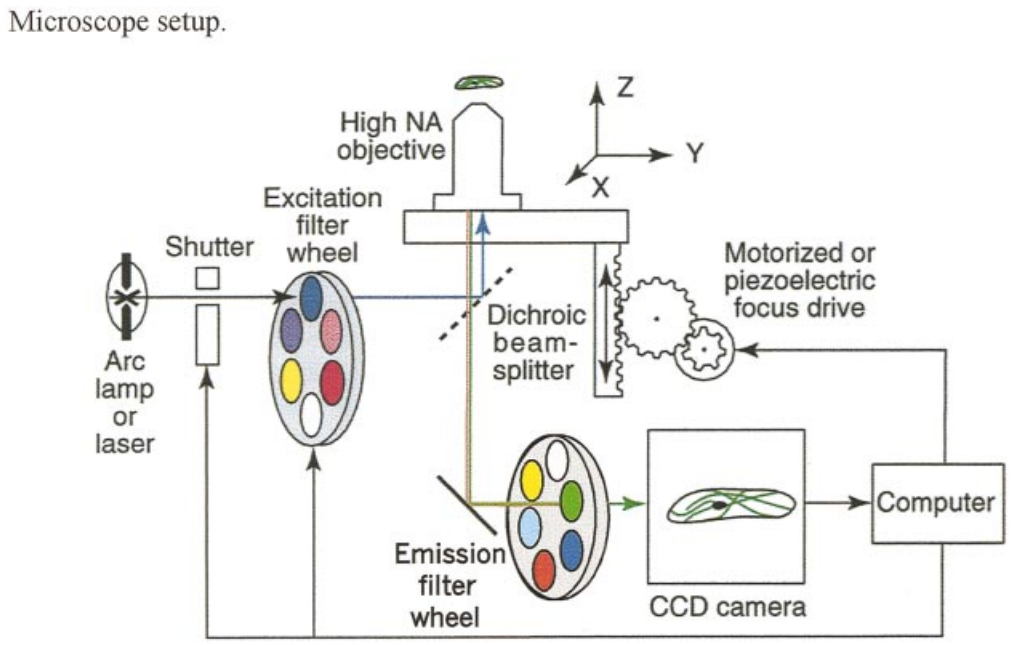

Figure 6 Microscope set-up for detection of GFP

A digital imaging system, built on an epifluorescence microscope, is equipped with filter-wheels placed on the excitation and emission light paths, a piezoelectric motor and a CCD camera. The system is operated by software that also permits analysis and computational deblurring of the images. NA, numerical aperture.

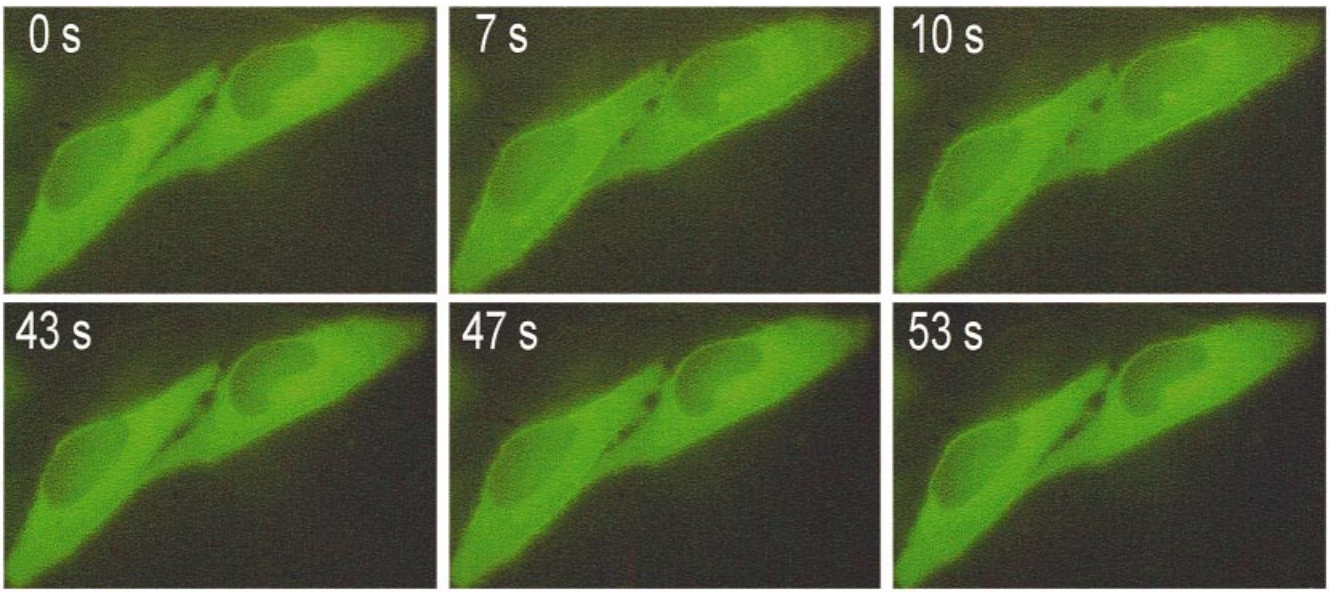

Figure 7 Time course of cellular distribution of PKC-β_{II}

The transient expression of PKC-β_{II}–HA1–eGFP in HeLa cells enables the pulse of this protein to be tracked between the cytosol and the plasma membrane, after the application of histamine (100 μM). The peaks at 7 s and 47 s are the clearest ones: after 1 min PKC β_{II} is again fully cytosolic, and it can be recruited again by new agonist stimulation (not shown). Time-lapse movies are available at: http://www.BiochemJ.org/bj/355/bj3550001add.htm

data to computer storage. A high-resolution image restoration of a limited volume of a cell has been obtained using a few as between five and seven optical sections [63], hence minimizing the total acquisition time.

A high-speed version of this microscopy has been developed [25,64] that can acquire an entire through-focus image series of a GFP-labelled cell in less than 1 s. This microscope system can be used to follow spatial and temporal intracellular dynamics (e.g. motor-protein based transport, signal transduction) that are too rapid for conventional fluorescence microscopy [25,65,66]. Conversely, if only the time course, rather than the three-dimensional distribution, of a fast process (e.g. Ca^{2+} signalling)

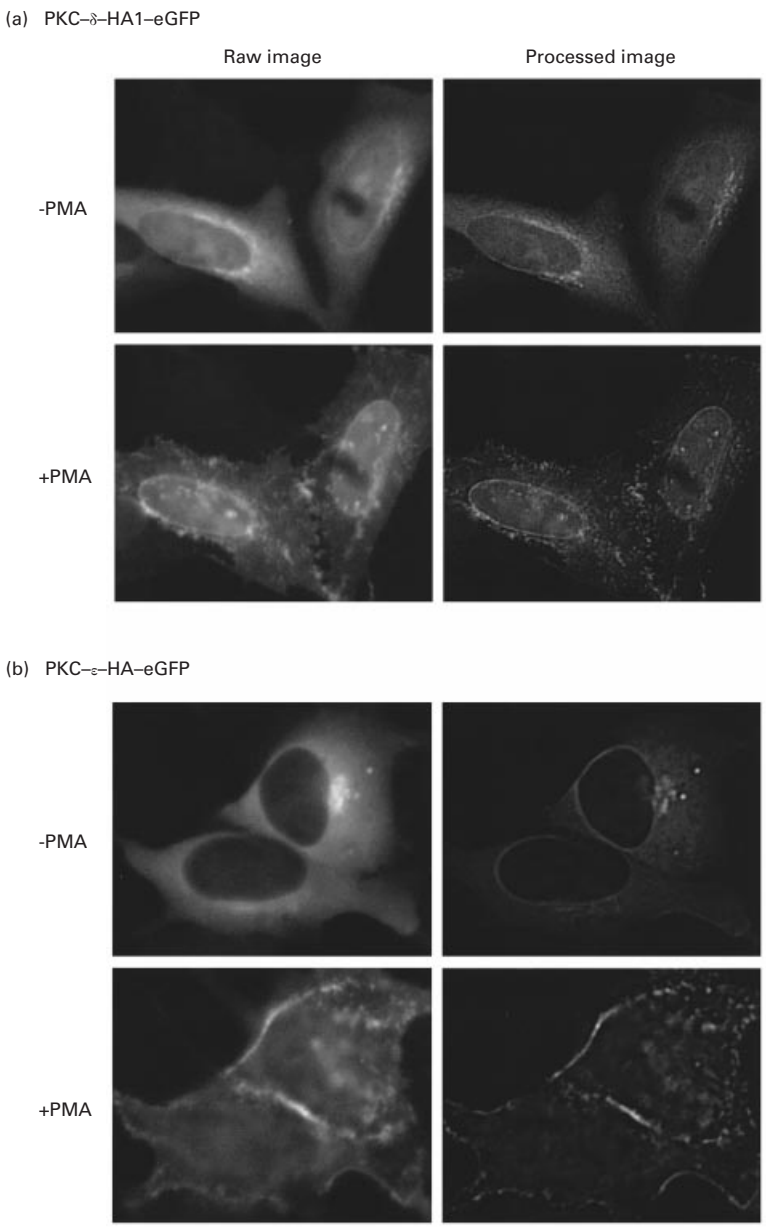

Figure 8 Cellular distribution of novel PKC isoforms

(**a**) PKC-δ–HA1–eGFP is localized in the cytosol and in the Golgi apparatus, but after treatment with PMA (300 nM) it translocates to the plasma membrane and to the nuclear membrane. (**b**) PKC-ε–HA1-eGFP is localized in the cytosol, in the Golgi apparatus and in the nuclear membrane; after PMA treatment this isoenzyme translocates only to the plasma membrane.

needs to be assessed, single fluorescent images of the microscope field of interest can be acquired every 10–20 ms with no further image processing [67].

Finally, the filter-wheels allow the alternate imaging of two different fluorophores at different excitation and emission wavelengths, and thus the simultaneous visualization of two different proteins of interest in the same cell (see the example in Figure 9 below), or the measurement of donor and acceptor fluorescence in FRET applications. When compared with laser scanning confocal microscopy, digital imaging is characterized by greater flexibility in the selection of excitation wavelengths, lower illumination intensity (thus reducing photobleaching and photodamage) and lower cost. Conversely, its disadvantages are the need for time-consuming off-line image processing, and unsuitability for the analysis of thick specimens (e.g. tissue slices).

Decoding the calcium signal: GFP as a probe of PKC recruitment

The PKCs are a family of serine/threonine kinases that play a major role in determining cellular responses to agonists coupled, through G_q proteins, to the production of inositol 1,4,5-trisphosphate (and hence the release of Ca^{2+} from the ER) and diacylglycerol [9,68]. These effector systems show a wide degree of molecular and biochemical heterogeneity: numerous isoforms of PKC have been identified, often co-expressed in the

same cell, that are classified in subgroups, based on sequence identity and sensitivity to activators [69]. The 'classical' isoforms, such as α, β_I, β_{II} and γ, are activated by increases in Ca^{2+} and diacylglycerol concentrations. The 'novel' isoforms, such as ϵ, δ, θ and η, are sensitive to diacylglycerol, but not to Ca^{2+}. The more recently identified 'atypical' isoforms (including ζ and λ) are insensitive to both diacylglycerol and Ca^{2+}, and their mode of activation is still largely unclear [70,71]. As to cell distribution, translocation to the cell membranes is, for all PKCs, a key step in the activation process. Indeed, ligand binding does not induce significant changes in protein conformation, but caps a hydrophobic site at the top of the structure, forming a contiguous hydrophobic region that promotes insertion of the 'cysteine-rich domain' into the lipid bilayer. Thus, while inactive PKCs are present mainly in the cytosol, activated PKCs are found associated with the plasma membrane, the nucleus and other subcellular compartments [72].

In this scenario, dissecting the signalling pathways in which PKCs are involved is a difficult task, given the overlapping substrate specificity and the complexity of the molecular machinery that regulates the specific sorting of the various isoforms (including scaffolding/adapter proteins that form multienzyme complexes with kinases and phosphatases). To obtain a deeper insight into these signalling events, we decided to construct a panel of GFP chimaeras, allowing us to monitor, in real time, the recruitment of the various PKC isoenzymes, an approach described previously by Meyer and co-workers [73]. We have employed two spectral variants of GFP: the S65T mutant, endowed with green fluorescence, and the Y66H/Y145F mutant (eBFP), which emits blue light upon UV illumination. Each PKC's cDNA (coding for isoforms α, β_{II}, δ, ϵ and ζ) was fused to HA1–eGFP or HA1–eBFP (where HA1 is the epitope derived from haemagglutinin, and eGFP and eBFP are the 'humanized' mutants of wild-type GFP). When expressed in mammalian cells, each chimaera was brightly fluorescent, endowing the cell with the expected fluorescence pattern.

Figures 7 and 8 provide representative examples of the use of some of the chimaeras. In Figure 7, the cell distribution of a classical PKC isoform (β_{II}) is shown. Several images are shown, taken at different times after the addition of histamine, an agonist coupled to the generation of Ins(1,4,5)P_3 (and thus the release of Ca^{2+} from intracellular stores) and diacylglycerol. It is apparent that, in parallel with increases in $[Ca^{2+}]_c$ (not shown), part of the PKC β_{II} pool translocates to the plasma membrane. In Figure 8, the distribution of PKC chimaeras of the novel class is investigated (δ in Figure 8a; ϵ in Figure 8b). Two sets of data are displayed: the distribution at rest and that after treatment with the phorbol ester PMA. For each experimental condition, two images are shown, corresponding to the three-dimensional reconstruction of the raw and of the computationally deblurred images respectively. It is apparent that, both at rest and upon stimulation, two isoenzymes belonging to the same class and exhibiting comparable biochemical properties show distinct patterns of subcellular distribution (of interest is the nuclear localization of the δ isoform).

Using GFP as a reporter of physiological parameters and/or enzymic activities

As mentioned above, following the seminal paper by Miyawaki and co-workers on CaMeleons [57], interest has focused on the development of fluorescent probes for cellular events of interest based on FRET between GFPs. Although the search has proven laborious (and in some cases quite frustrating), various successful examples are now available, ranging from the measurement of cAMP levels to the interaction of transcription factors on gene promoters [74]. Here we will describe an application developed by various groups [54,55], including our own: the use of GFP for monitoring the activation of caspases, the proteases involved in programmed cell death. In brief, a chimaera has been constructed in which a GFP moiety, the green mutant GFP(S65T), acting as acceptor fluorophore, has been fused to a GFP moiety acting as donor, the Y66H/Y145F blue mutant, via a linker sequence including a short consensus (DEVD) recognized by a subset of caspases (caspase 3-like). *In vitro* experiments demonstrated that incubation of the 'DEVD sensor' with a specific protease abolishes FRET between the two GFPs. Figure 9 shows the emission spectrum of the fluorescent protein before (green) and after (red) proteolytic cleavage of the linker. The blue emission of eBFP is enhanced, while the green emission of eGFP is greatly decreased. Control experiments in the same conditions applied to either GFP mutant alone had no effect (results not shown). We are now testing the same fusion protein *in vivo*, and our goal is to construct targeted variants of the caspase sensor to investigate the subcellular pattern of caspase activation in the course of apoptosis.

Figure 9 Emission spectra of the fusion protein eBFP–DEVD–eGFP

Two peaks are evident: at the eBFP peak 445–450 nm and the eGFP peak at 511 nm. Excitation was at 380 nm. The green and red curves were obtained before and after incubation with the protease respectively. Inversion of the emission spectra, reflecting the abolition of FRET between the two fluorophores, is apparent.

CONCLUSIONS

In this review, we have summarized our use of two recombinant proteins, aequorin and GFP, in cell biology studies. These proteins are well tolerated by the cell and may be expressed at high levels in numerous heterologous systems. Aequorin is a good probe with which to follow the kinetics of calcium signals in defined cellular compartments, and thus has proved instrumental in revealing the participation of organelles in calcium homoeostasis. GFP, as a genetically encoded fluorescent molecule (which can be expressed in cells with minimal toxicity and fused to resident proteins without affecting their structure or biochemical properties), has a number of important applications in the study of cell signalling. It allows the unambiguous identification of subcellular structure, and the tracking of signalling molecules. Furthermore, through the phenomenon of FRET, it may allow the development of novel probes for measuring physiological parameters.

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