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# Engineering firefly luciferase as an indicator of cyclic AMP-dependent protein kinase in living cells

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A bioluminescent indicator for protein kinase A has been developed by mutating V217 in firefly (*Photinus pyralis*) luciferase to R, and the C-terminal peroxisomal signal removed by PCR. The cDNA for normal and the RRFS mutant luciferase were inserted into pSV7d and expressed in COS-7 cells. Transient expression in approximately 5% of cells was confirmed by extraction of active luciferase, light emission from cells in the presence of luciferin, and immuno-localisation. The cyclic-AMP analogue, 8-(4-chlorophenylthio)-cyclic AMP caused a 5–10% decrease in light emission within 4 min in COS cells expressing the RRFS mutant, but not in cells expressing normal luciferase. This provides for the first time an indicator for detecting and quantifying protein kinase A activation in living cells.

Cyclic AMP; Protein kinase; Luciferase; Bioluminescence

## 1. INTRODUCTION

It is well established that both cyclic AMP and cyclic GMP play a key role in signalling cell activation, division and development, and defence [1,2]. The kinases and phosphatases which are activated by these intracellular signals have also been well characterised [3]. cAMP binds to the regulatory subunit (R) of $C_2R_2$, releasing the catalytic subunit which then binds to particular sites on targeted proteins. A key recognition sequence in these proteins is RRXS, although residues on either side of this can affect the selectivity and affinity for the kinase [3,4]. Yet precisely how protein kinase A is involved in many types of cell activation, particularly in the control of gene expression required for cell division or transformation, is not well established [1].

Understanding of the role of $Ca^{2+}$ in cell activation and cell injury has been revolutionised by the technology for measuring and manipulating free $Ca^{2+}$, and for locating it, in live cells [5–8]. A major problem in establishing definitively the role of protein kinase A in a cellular event has been the lack of methods for measuring and locating protein phosphorylation in living cells. We have recently established a strategy using engineering of bioluminescent proteins to solve this problem [9,10]. cDNA coding for firefly luciferase was engineered using PCR to contain a protein kinase A recognition site, RRFS [10], and lacking the C-terminal peroxisomal signal in native luciferase [11]. Phosphorylation by protein kinase A caused an 80–90% decrease in activity in vitro, which was reversed by addition of phosphatase. Here we report the expression of this variant in COS cells, and, for the first time, the detection of cyclic AMP-activated kinase in live cells.

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## 2. EXPERIMENTAL

The T7 RNA polymerase promoter was added to the 5′ end of firefly luciferase cDNA, cloned in pcDV1 primer and Honjo linker as previously described [10], a *Sal*I site to the 3′ end and the codons for the last three amino acids, SKL, the peroxisomal signal [11], removed by PCR using a 3′ anti-sense primer CTGCTTGAGCTCGTCGACTTACTTTCCGCCCTTCTTG. The codon for valine at position 217, GTC, was mutated to the codon for arginine, CGC, by two stage PCR as previously described [10], generating a site coding for RRFS. Recombinant normal and mutant (RRFS) firefly luciferases were generated by first transcribing the PCR DNA product using T7 RNA polymerase, and then translating the capped mRNA in rabbit reticulocyte lysate, optimised for $K^+$ and $Mg^{2+}$ [9]. Inhibitory factors to the catalytic subunit of protein kinase A were removed by gel filtration. The activities of the recombinant proteins were determined by measuring chemiluminescence counts per 10 s in a buffer containing 20 mM Tris-acetate, 12 mM magnesium acetate, 0.3 mM dithiothreitol, 0.1% w/v bovine serum albumin and 200 μM luciferin, pH 7.8. Specific activities were determined by relating light emission to ng protein measured from [$^{35}$S]methionine incorporation [10]. Chemiluminescence was measured in a home-built chemiluminometer [12].

The PCR DNA products were cut with the restriction enzyme, *Sal*I, and ligated into pSV7d [13] cut with restriction enzymes, *Sma*I and *Sal*I. The plasmid ligation mixture was used to transform *E. coli* K12-MC1061 strain using $CaCl_2$ and heat shock [14]. Colonies growing on ampicillin plates were screened for luciferase cDNA inserts by PCR, with 1 in 30–40 colonies containing inserts. Insertion was established by the size of the new plasmid (approx. 2,423 + 1,650 bp), and the correct orientation downstream from the SV40 early promoter confirmed by restriction endonuclease digestion with *Bsc*I, II or *Sal*I. The activity of the insert from single clones was checked using in vitro transcription–translation, to ensure that the PCR followed by subcloning had selected a fully active luciferase. Mutant RRFS luciferase

had a specific activity (CL counts/ng protein) which was only 15% that of normal luciferase [10]. Removal of SKL from the C-terminus appeared to increase the specific activity of luciferase, in contrast to removal of the last 12 amino acids which virtually abolished activity [9]. Incubation of the RRFS mutant, synthesised from the pSV7d plasmid, with the catalytic subunit of protein kinase A reduced the activity of the RRFS luceriferase by 85%.

The pSV7d plasmids, $A_N$ (normal luciferase–SKL) and $C_3$ (RRFS luciferase–SKL), were then used to transfect COS-7 cells using calcium phosphate [14], with 1 μg of DNA added to each well containing approximately $7.5 \times 10^4$ cells. After 48 h in DMEM + 10% foetal calf serum, culture medium expression of luciferase was assessed by (i) freeze–thaw extraction and assay for luciferase, for which the specific activity of normal recombinant luciferase was 260,000 CL counts/10 s/ng protein and mutant RRFS luciferase was 30,000 CL counts/10 s/ng protein, (ii) light emission detected at 20°C in a home-built X-Y table microtitre plate chemiluminometer, after addition of 1 mM luciferin (cross-talk was negligible, i.e. less than 4% between adjacent wells), and (iii) immunofluorescence with antibody raised in rabbits to firefly luciferase.

## 3. RESULTS AND DISCUSSION

Immunofluorescence showed that approximately 5% of the transfected COS cells expressed luciferase (Fig. 1). This agreed well with other reports [15,16]. The staining was diffuse, suggesting that the majority of the expressed recombinant protein was cytosolic, as predicted for luciferase lacking the native peroxisomal signal [11]. Other workers who have expressed firefly luciferase cDNA in eukaryotic cells have not removed this peroxisomal signal, and have not fully established the cytosolic location of their expressed protein.

Addition of luciferin to the cells resulted in light emission almost immediately. Cells expressing the RRFS mutant produced some 10-times less light per microtitre well, consistent with the reduced specific activity of this variant [10], however, the time–course of light emission was highly variable between experiments (Fig. 2). Decreases and increases in light emission with time, as well as stable light emissions, were observed in various experiments. The cause of this variability is not known. Extraction of both normal and RRFS luciferases from COS cells, followed by assay in an optimal medium, resulted in approximately 5-times more light emission than that measured directly from the live cells. Light emission from cells expressing normal luciferase–SKL was $11 \pm 7 \times 10^6$ CL counts/$10^6$ cells (mean ± S.D. of 3 experiments) compared with $54 \pm 35 \times 10^6$ CL counts/$10^6$ cells for luciferase extracted from the cells. This was consistent with the 29% inhibition of luciferase activity

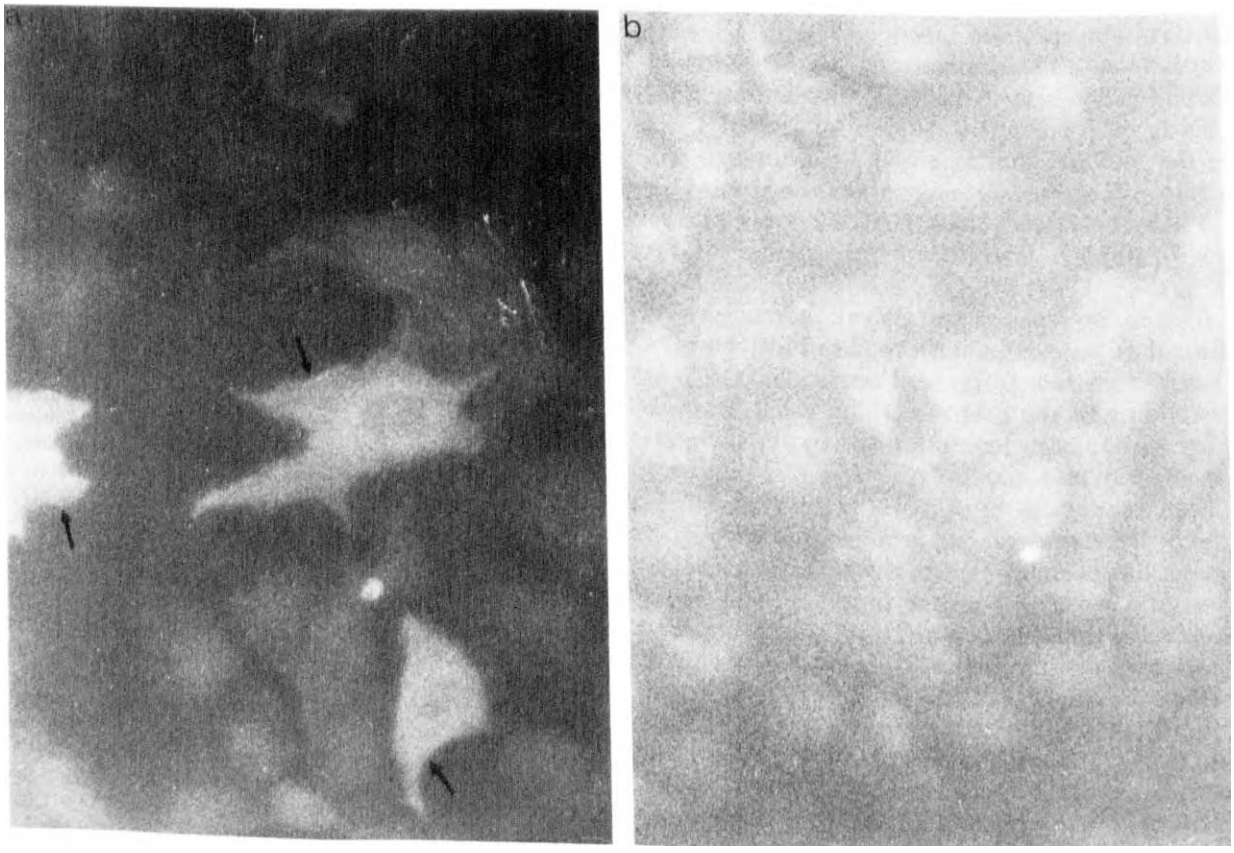


Fig. 1. Expression of luciferase cDNA in COS cells detected by immunofluorescence. COS-7 cells grown on cover-slips were transfected with CsCl-purified plasmid pSV7d-$A_n$ DNA (a) or left untransfected (b). After 48 h the cells were fixed in 4% v/v formaldehyde, permeabilised with 0.1% v/v nonidet NP40 in 50 mM sodium phosphate, 150 mM NaCl, 10% v/v sheep serum, pH 7.4 [14] and stained using rabbit anti-luciferase serum (1/100 dilution) followed by fluorescein-labelled goat anti-rabbit IgG as second antibody. Arrows indicate cells expressing luciferase (a), clearly visible over the background fluorescence of untransfected cells (b).

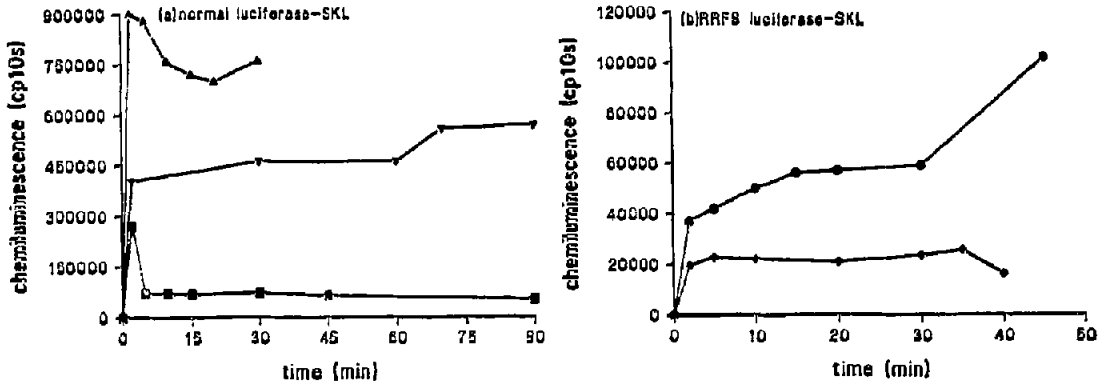


Fig. 2. Variable light emission from COS cells expressing normal and RRFS luciferase cDNAs. Transfected cells expressing normal luciferase-SKL (a) and the RRFS mutant-SKL (b) were grown in 24-well microtitre plates. After 48 h the culture medium was replaced by Krebs–Ringer–HEPES medium [6] containing 0.1% (w/v) bovine serum albumin and 1 mM luciferin at 20°C. Chemiluminescence was measured using a home-built chemiluminometer coupled to a computer-operated X-Y table. Traces represent examples of five different cell preparations.

found in the synthetic intracellular medium: 100 mM potassium glutamate, 10 mM NaCl, 10 mM magnesium acetate, 1 mM sodium phosphate, 10 mM MES, 10 mM Tris, 0.1% bovine serum albumin, 0.4 mM EDTA, pH 7.2. Also the oxyluciferin is a potent inhibitor, which is alleviated in vitro by pyrophosphate. Other cellular constituents such as acetyl CoA and pH may also affect the activity, the latter causing the emission to shift to the red at acid pH, resulting in an apparent loss of light because of the spectral sensitivity of the photomultiplier tube [12].

Nevertheless reproducible, small effects of the cyclic AMP analogue 8-(4-chlorophenylthio)-cyclic AMP on light emission from cells expressing the RRFS mutant were detected (Fig. 3a). Within 4 min of addition of the cyclic AMP analogue (100 $\mu$M) the light emission from RRFS cells decreased by approximately 10% relative to control cells, where buffer alone was added. This implied that the new balance between activated kinase A and phosphatase was achieved within 4 min, since the traces ± cyclic AMP were parallel for the remainder of the experiment (60 min). No effect of the cyclic AMP analogue was seen in cells expressing normal luciferase (Fig. 3b). The question now arises as to why the effect on RRFS activity was so small. In vitro addition of the catalytic subunit of protein kinase A caused an 80–90% decrease in the activity of the RRFS luciferase [10]. Although the luciferase was distributed throughout the cell (Fig. 1), the intracellular distribution of protein kinase A in these cells is not known. Thus it will now be necessary to image [16] the kinase signal in individual cells to define how many cells actually responded and where within the cell the kinase activation occurred.

These results show for the first time the detection of kinase activation in live cells. The ability to target such bioluminescent indicators to defined sites within cells [16] opens up a new era for the measurement of intracellular signalling in living cells.

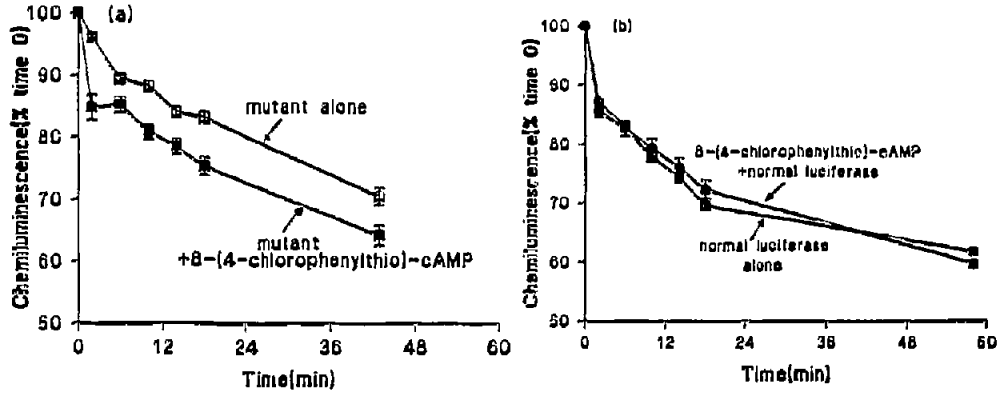


Fig. 3. Effect of 8-(4-chlorophenylthio)-cyclic AMP on light emission from RRFS mutant luciferase expressed in COS cells. Cells expressing RRFS mutant luciferase-SKL (a) and normal luciferase-SKL (b) were grown in microtitre plates and incubated with 1 mM luciferin, as described in Fig. 2. After 10 min 100 $\mu$M 8-(4-chlorophenylthio)-cyclic AMP was added (■,●) and the light emission expressed as a % of time 0 (i.e. after addition of the cAMP analogue) compared with cells incubated without cAMP (□,○). Final number of cells per well = $3 \times 10^5$. Results represent the mean ± S.E.M. of three wells.

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