A colourless green fluorescent protein homologue from the non-fluorescent hydromedusa *Aequorea coerulescens* and its fluorescent mutants

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We have cloned an unusual colourless green fluorescent protein (GFP)-like protein from *Aequorea coerulescens* (acGFPL). The *A. coerulescens* specimens displayed blue (not green) luminescence, and no fluorescence was detected in these medusae. *Escherichia coli* expressing wild-type acGFPL showed neither fluorescence nor visible coloration. Random mutagenesis generated green fluorescent mutants of acGFPL, with the strongest emitters found to contain an $Glu^{222} \longrightarrow Gly$ (E222G) substitution, which removed the evolutionarily invariant Glu^{222}. Re-introduction of Glu^{222} into the most fluorescent random mutant, named aceGFP, converted it into a colourless protein. This colourless aceGFP-G222E protein demonstrated a novel type of UV-induced photoconversion, from an immature non-fluorescent form into a green fluorescent form. Fluorescent aceGFP may be a useful biological tool, as it was able to be expressed in a number of mammalian cell lines. Furthermore, expression of a fusion protein of 'humanized' aceGFP and β-actin produced a fluorescent pattern consistent with actin distribution in mammalian cells.

Key words: bioluminescence, green fluorescent protein (GFP), photoconversion, protein labelling.

INTRODUCTION

Green fluorescent protein (GFP) from the hydromedusa *Aequorea victoria* is intensively used in biomedical sciences [1]. In nature, GFP is a component of the *A. victoria* bioluminescent system. Like many other marine organisms, this jellyfish can produce bright flashes in response to external stimulation. GFP is a secondary emitter that transforms blue light (460 nm), emitted by the Ca^{2+}-dependent photoprotein aequorin, into green light (508 nm) [2]. Although GFPs similar to *Aequorea* GFP are found in other bioluminescent coelenterates (for review see [3]), the biological significance of blue light transformation is not clear, particularly since the majority of bioluminescent animals do not possess this mechanism.

The discovery of GFP-like proteins in the non-bioluminescent Anthozoa species indicates these proteins are not necessarily linked to bioluminescence [4]. In addition, an interesting group of non-fluorescent GFP-like chromoproteins has been characterized [5–7]. Fluorescent proteins in corals are believed to be photoprotective [8], although this function does not appear to explain the diversity of GFP-like proteins in Anthozoa [9,10].

The known GFP homologues can be divided into four main colour groups: green, yellow and orange–red emitters and non-fluorescent purple–blue chromoproteins. In the present work we describe an unusual colourless GFP homologue from *A. coerulescens*. Despite very high sequence similarity to *A. victoria* GFP, this novel protein possesses unique spectral properties.

EXPERIMENTAL

Gene cloning and mutagenesis

Total RNA was isolated from a small piece of a single specimen of *A. coerulescens*, which included the umbrella border and radial channel, using a NucleoSpin RNA II kit (ClonTech). cDNA was synthesized and amplified with a SMART PCR cDNA Synthesis kit (ClonTech). A fragment of the novel GFP homologue cDNA was obtained by performing PCR with degenerate primers using a strategy described previously [4]. The step-out PCR rapid amplification of cDNA ends ('RACE') method [11] was used to clone the 5′-end region of the target cDNA. The cDNA nucleotide sequence encoding the novel GFP-like protein from *Aequorea coerulescens* (acGFPL) has been deposited in the GenBank® database under the accession number AY151052. For the bacterial expression of acGFPL, the full-length coding region was amplified using specific primers and cloned into the pQE30 vector (Qiagen).

The Diversity PCR Random Mutagenesis kit (ClonTech) was used for random mutagenesis of acGFPL, under conditions optimal for 5–6 mutations per 1000 bp. *Escherichia coli* colonies expressing mutant proteins were visually screened with a fluorescent stereomicroscope SZX-12 (Olympus). The brightest variants were selected and subjected to another round of random mutagenesis. Site-directed mutagenesis was performed by PCR using the overlap extension method, with primers containing appropriate target substitutions [12].

Protein expression and spectral measurements

Proteins fused to an N-terminal His_6 tag were expressed in *E. coli* XL1 Blue strain (Invitrogen) and purified using TALON metal affinity resin (ClonTech). Absorption spectra were recorded with a Beckman DU520 UV/VIS spectrophotometer. A Varian Cary Eclipse fluorescence spectrophotometer was used for measuring excitation–emission spectra.

To determine the molar absorption coefficient, we relied on estimating mature chromophore concentration. Proteins were

Abbreviations used: GFP, green fluorescent protein; acGFPL, GFP-like protein from *Aequorea coerulescens*; EGFP, enhanced GFP; ace-GFP, most fluorescent green mutant protein; G222E, $Gly^{222} \longrightarrow Glu$ substitution; aceGFP-h, 'humanized' aceGFP.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AY151052 and AY233272.

denatured under alkali conditions with an equal vol. of 2 M NaOH. Under these conditions, the GFP chromophore absorbs at 446 nm and its molar absorption coefficient is 44 000 $M^{-1} \cdot cm^{-1}$ [13]. Absorption spectra for native and alkali-denatured proteins were measured. Molar absorption coefficients for native state proteins were estimated based on the absorption of denatured proteins. For quantum yield determination, the fluorescence of mutants was compared with equally absorbing enhanced (E)GFP (quantum yield 0.60 [14]).

UV-induced photoconversion of aceGFP-G222E (the most fluorescent green mutant with an $Gly^{222} \longrightarrow Glu$ substitution) was performed using the Varian Cary Eclipse fluorescence spectrophotometer. The protein sample was irradiated for several hours with 250–300 nm light in scanning mode (excitation slit 20 nm, scan rate 30 nm/min, averaging time 1 s, cycle mode).

Gel filtration

Purified protein samples (approx. 1 mg/ml) were loaded on to a Sephadex-100 column (0.7 cm × 60 cm) and eluted with a solution of 50 mM phosphate buffer (pH 7.0) and 100 mM NaCl. EGFP, HcRed1 and DsRed2 (ClonTech) were used as monomer, dimer and tetramer standards respectively.

SDS/PAGE and Western blotting

Heated and unheated samples in a standard reducing sample buffer were subjected to SDS/PAGE (12 % gel) at 15 mA/gel. For Western blotting, proteins were transferred on to Hybond C membranes (Amersham) using standard procedures. Membranes were probed with mouse antibodies (ClonTech) against GFP (1 : 2500 dilution), and then with HRP-conjugated anti-mouse antibodies (Amersham) at 1 : 2500 dilution. Positive signals were visualized using the ECL® Western-blotting analysis system (Amersham Pharmacia Biotech), involving Detection reagents 1 and 2, and Hyperfilm ECL.®

Expression in mammalian cell lines

For expression in eukaryotic cells, aceGFP and its 'humanized' version, aceGFP-h, were cloned into pEGFP-C1 and pEGFP-Actin vectors (ClonTech) between *Age*I and *Bgl*II restriction sites (in lieu of the EGFP-coding region). The following cell lines were used: 293T human kidney epithelial cells, 3T3 mouse embryo fibroblasts, L929 murine subcutaneous fibroblasts, Vero African green monkey kidney epithelial cells and COS-1 African green monkey kidney fibroblasts. Cells were transfected using LIPOFECTAMINE™ reagent (Invitrogen) and were tested 20 h after transfection. An Olympus CK40 fluorescence microscope equipped with a charge-coupled-device camera (DP-50, Olympus) was used for cell imaging.

RESULTS

Description of the medusa *A. coerulescens*

Several specimens of large hydromedusa were collected from the Russian coast of the Japan Sea near Vladivostok in August 2001. A set of characteristic features allowed us to define these medusae as *A. coerulescens* [15,16]. Although *A. coerulecens* and *A. victoria* (other names for the latter are *A. forscalea* and *A. aequorea*) look similar, some of their features are clearly different. The most obvious difference is that *A. victoria* carries only 1 tentacle per radial channel, whereas *A. coerulescens* possesses 4–6 tentacles between each pair of adjacent radial channels [15].

In contrast to *A. victoria*, *A. coerulescens* medusae displayed blue, not green, luminescence. We cloned the aequorin-like photoprotein apparently responsible for the bioluminescence observed in *A. coerulescens* specimens (GenBank® accession number AY236998). This molecule shared 84 % identity at the amino acid level with *A. victoria* aequorin, and had an emission maximum at 455 nm. No detectable fluorescence was observed in *A. coerulescens* under either UV light or using a fluorescence microscope. However, using monoclonal antibodies against GFP we found that a protein extract from *A. coerulescens* contained a GFP-like protein (see Figure 3A).

Cloning of cDNA encoding a GFP-like protein from *A. coerulescens*

To search for GFP-like proteins in *A. coerulescens*, we employed a PCR-based strategy using degenerate primers corresponding to conservative amino-acid sequences [4]. As a result, cDNA encoding a GFP homologue was cloned and given the name acGFPL. The predicted novel protein was very similar to GFP, with 92 % amino acid identity (Figure 1). All known key GFP residues are conserved in acGFPL, including the chromophore-forming Ser^{65}, Tyr^{66} and Gly^{67} residues, the evolutionarily invariant Arg^{96} and Glu^{222}, and His^{148}, Phe^{165}, Ile^{167} and Thr^{203} which are all spatially close to the chromophore. Only three buried amino acids differed between the GFP proteins.

Taking into account the high sequence similarity, we expected that spectral properties of acGFPL would be similar to that of GFP. However, *E. coli* colonies expressing acGFPL showed neither fluorescence nor coloration. Incorrect folding of acGFPL in *E. coli* was found to only partially explain these observations (see below).

acGFPL cDNA distribution throughout the medusae was tested using PCR with specific primers. acGFPL cDNA was clearly detected in the umbrella border, but not in the radial channel or oral disc samples. Thus distribution of acGFPL within the jellyfish body appears similar to that of GFP, which forms a fluorescent ring in the *A. victoria* umbrella border.

Random mutagenesis

Random mutagenesis was used to improve protein folding and to generate fluorescent mutants of acGFPL. After the first round of mutagenesis several green fluorescent colonies of different brightness were found. The three brightest clones (named G1, Z1 and Z2) were further characterized and the mutants were found to contain the same substitution, E222G. Additional V11I/K101E and N19D mutations were found in G1 and Z2 respectively. Thus substituting Glu^{222} appeared to be the key event in creating a fluorescent form of acGFPL.

The spectra of the three mutants appeared to be similar. Excitation spectra were single peaked at 475 nm (excluding the peak at 280 nm) and the emission maxima were at 505 nm. Absorption spectra demonstrated complete absence of the form absorbing at 390 nm.

Two additional rounds of random mutagenesis on the G1 clone (only G1 was able to mature at 37 °C, whereas Z1 and Z2 required incubation at low temperature to develop fluorescence) generated a very bright mutant, named aceGFP, which matured fast at 37 °C. This mutant contained five amino-acid substitutions compared with the wild-type acGFPL, specifically V11I, F64L, K101E, T206A and E222G (Figure 1). Like the parental clones, aceGFP possessed a single excitation maximum at 480 nm and

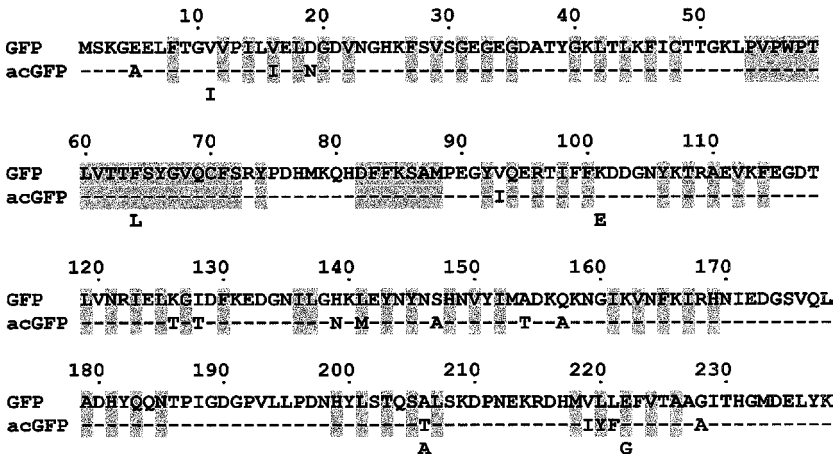

Figure 1 Alignment of GFP and acGFPL amino acid sequences

acGFPL residues identical with the corresponding amino acids in GFP are represented by dashes. Buried residues that form the interior of the β-can are shaded in grey. aceGFP amino-acid substitutions are shown under the acGFPL sequence.

an emission maximum at 505 nm (Figure 2A). pH titration in the acidic region (pH 5.0 and below) demonstrated gradual transformation of the 480 nm absorption peak into the peak at 386 nm with an isosbestic point at 418 nm (results not shown). Acid- and alkali-denatured aceGFP possessed absorption maxima at 380 and 446 nm respectively. These results, together with the high sequence similarity, clearly indicate that aceGFP carries a chromophore identical with that of *A. victoria* GFP in the deprotonated state. A molar absorption coefficient of 50 000 $M^{-1} \cdot cm^{-1}$ and a quantum yield of 0.55 make aceGFP almost as bright as the widely used EGFP [14]. Gel-filtration tests (results not shown) and SDS/PAGE of non-heated proteins [17] demonstrated that aceGFP is monomeric (Figure 3).

Mutagenesis at position 222

To clarify the importance of the E222G substitution for fluorescence, we applied a reverse G222E substitution on to the optimal mutant aceGFP. Unlike the colonies expressing aceGFP, *E. coli* colonies expressing aceGFP-G222E displayed neither coloration nor detectable fluorescence under fluorescence microscopy following excitation at around 400 and 480 nm.

aceGFP-G222E comprised approx. 20% of total bacterial protein, indicating this soluble recombinant protein was efficiently expressed and folded in *E. coli* at 37 °C. The colourless purified aceGFP-G222E had an absorption spectrum with a major peak at 280 nm and a minor peak at 390 nm (Figure 2B). Alkali-denatured aceGFP-G222E showed an absorption peak at 446 nm, similar to the anionic form of the GFP chromophore. Assuming the chromophore absorption coefficient is 44 000 $M^{-1} \cdot cm^{-1}$ [13], we estimated the absorption coefficient of native aceGFP-G222E at 390 nm to be 33 000 $M^{-1} \cdot cm^{-1}$. The observed ratio between 280 nm and 390 nm peaks (molar absorption coefficient at 280 nm was calculated to be 23 500 $M^{-1} \cdot cm^{-1}$ [18]) showed that only approx. 3% of soluble aceGFP-G222E existed in a mature form. Excitation at 390 nm led to a weak dual-colour fluorescence peaking at 460 nm and 505 nm, with a quantum yield of 0.07 (Figure 2C).

GFP-like proteins retain their spectral properties and oligomerized state under standard SDS/PAGE conditions if protein samples are not heated before loading on to the gel [17]. Such analysis of aceGFP-G222E demonstrated a clear difference in the mobility of non-denatured and denatured proteins (Figure 3A). In addition, the non-heated aceGFP-G222E protein band produced very weak fluorescence under UV light (Figure 3B). About 97% of this protein is present in a form with no light absorption, except the peak at 280 nm (Figure 2B), thus the conformation of this non-absorbing form is close to the native, not the denatured, state.

On the basis of the above results, we again attempted to obtain and characterize recombinant wild-type acGFPL. *E. coli* expressing acGFPL were grown at 20 °C without induction, and then the biomass was incubated for several days at 4 °C. This procedure resulted in the appearance of a small fraction of soluble acGFPL, comprising approx. 5% of total acGFPL. Absorption and fluorescence spectra for soluble wild-type acGFPL were very similar to that of aceGFP-G222E (results not shown). We concluded that aceGFP-G222E has the main properties of natural acGFPL, but possesses improved protein folding and temperature stability in *E. coli*.

These results showed that soluble aceGFP-G222E and wild-type acGFPL exist in two forms. The major form is folded, but immature without a spectrally detectable chromophore (the possible nature of this form is discussed below), whereas the minor 390 nm-absorbing form contains a GFP-like chromophore in a neutral state and possesses weak dual-colour fluorescence.

UV light-induced photoconversion

A novel type of photoconversion was observed in aceGFP-G222E. Irradiation of the protein with 250–300 nm UV light resulted in appearance of a 480 nm peak in the absorption/excitation spectra (Figure 4). This form apparently originates from some immature, spectrally undetectable form since the 390 nm absorption peak did not decrease during this photoconversion. Excitation at 480 nm produced green fluorescence at 505 nm with a high quantum yield (0.45). In these experiments, we achieved more than 1000-fold increase of the green fluorescence intensity (excitation at 480 nm).

Use of aceGFP as a fluorescent marker *in vivo*

To investigate the use of aceGFP as a fluorescent marker we examined protein expression in mammalian cells. Unexpectedly,

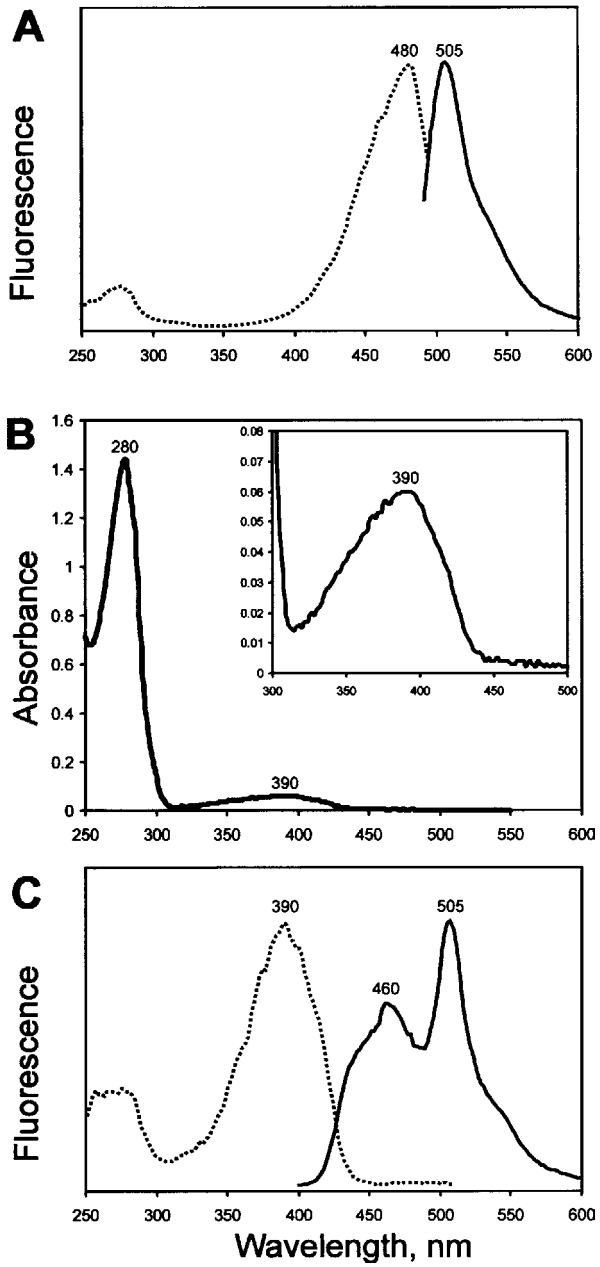

Figure 2 Spectral properties of acGFPL mutants

(**A**) Excitation (broken line) and emission (continuous line) spectra for the mutant aceGFP. (**B**) Absorption spectrum for the colourless mutant aceGFP-G222E. The inset magnifies the 300–500 nm region. (**C**) Excitation (broken line) and emission (continuous line) spectra for the mutant aceGFP-G222E.

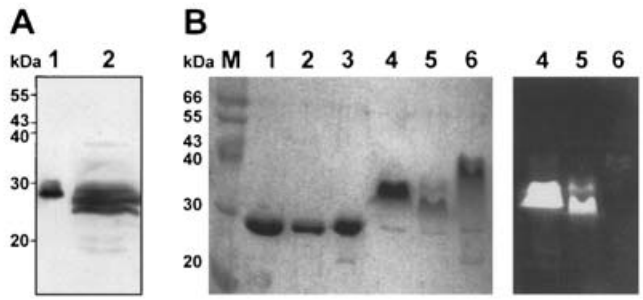

Figure 3 Protein gel electrophoresis analysis of acGFPL and its mutants

(**A**) Western-blot analysis of the soluble protein extract from *A. coerulescens* medusa using antibodies against GFP. Lane 1, purified recombinant *A. victoria* GFP; lane 2, *A. coerulescens* extract. The two major bands of about 27 and 25 kDa probably correspond to full-length acGFPL (predicted molecular mass is 26 896 Da) and a truncated acGFPL without seven N-terminal and 12 C-terminal residues (predicted molecular mass is 24 831 Da) that can be removed by proteases in the protein extract. Alternatively, presence of other GFP homologues is possible. (**B**) Comparison of the mobility of heated (lanes 1–3) versus non-heated (lanes 4–6) protein samples. Lanes 1 and 4, *A. victoria* GFP; lanes 2 and 5, aceGFP; lanes 3 and 6, aceGFP-G222E Lane M, molecular-mass markers. Coomassie Blue staining is shown in the left panel, fluorescence of the non-heated proteins under UV light is shown in the right panel.

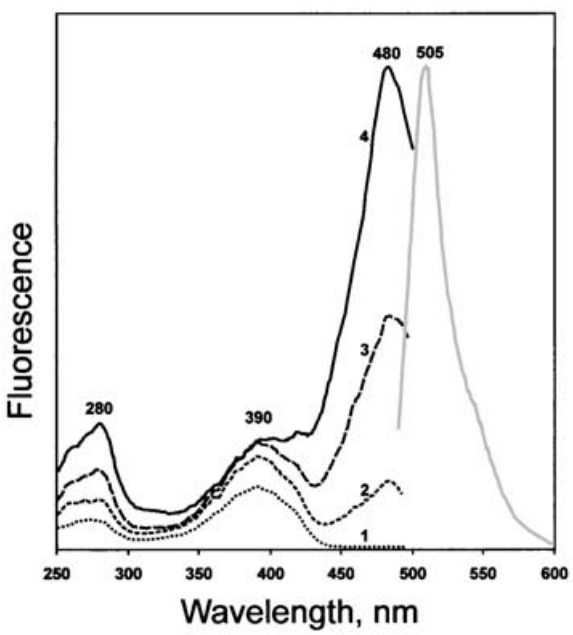

Figure 4 UV-induced photoconversion of aceGFP-G222E protein

Excitation spectrum (emission at 520 nm) of aceGFP-G222E before irradiation (line 1) and after irradiation with 250–300 nm light (lines 2–4). The grey line represents emission spectrum after photoconversion (excitation at 480 nm).

aceGFP produced very low fluorescent signals in human cell lines, suggesting either non-optimal codon usage or presence of a cryptic intron in the aceGFP gene. To overcome these possible problems we synthesized the aceGFP-h gene using mammalian-optimized codons (GenBank® accession number AY233272). This approach had a major effect, with transient expression of aceGFP-h in different cell lines producing bright green signals without aggregation (Figures 5A–5E). Fluorescence was clearly detectable 24 h after transfection. No cell toxicity was observed.

To determine the ability of aceGFP-h to tag proteins we constructed a fusion protein with cytoplasmic β-actin. Transient expression of the aceGFP-h–β-actin fusion protein in L929 cells produced a bright pattern consistent with actin labelling, with stress fibres, focal contacts and cell processes clearly visible (Figure 5F). There was no evidence of reduced cell adhesion, reduced cell viability or non-specific protein aggregation.

DISCUSSION

In the present work we have characterized an unusual colourless GFP-like protein. Our results clearly demonstrate acGFPL is a

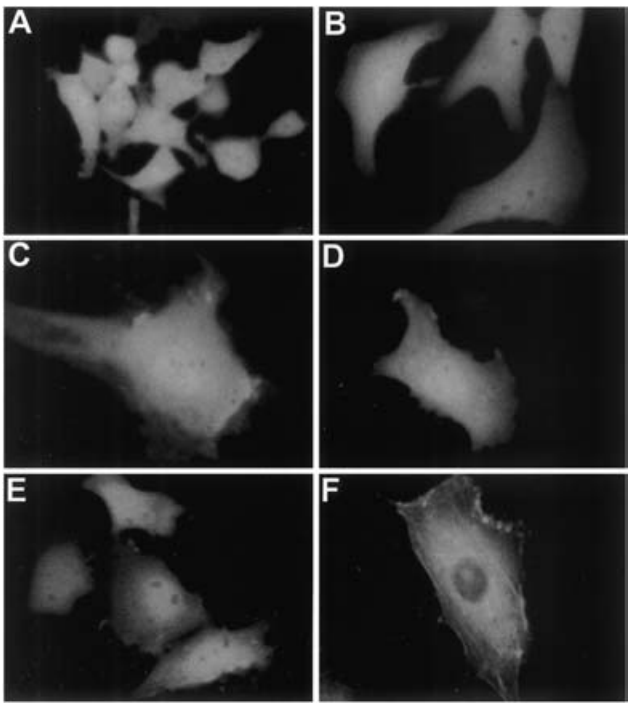

Figure 5 Expression of aceGFP-h and aceGFP-h–β-actin fusion protein in various mammalian cell lines

Cells were transiently transfected with constructs coding for aceGFP-h (**A**–**E**) and aceGFP-h–β-actin fusion protein (**F**). Cell types used were (**A**) 293T human kidney epithelial cells, (**B**) Vero African green monkey kidney epithelial cells, (**C**) 3T3 mouse embryo fibroblasts, (**D**) L929 murine subcutaneous fibroblasts, (**E**) COS-1 African green monkey kidney fibroblasts and (**F**) 3T3 mouse embryo fibroblasts.

natural product rather than an artefact, since *A. coerulescens* expressed acGFPL at both cDNA and protein levels. Furthermore, blue luminescence and lack of green fluorescence in *A. coerulescens* accords with the greatly diminished fluorescence of recombinant acGFPL. This protein mostly exists in a non-absorbing form, and the minute amount excitable at 390 nm is unable to accept energy from aequorin. The non-absorbing mutant aceGFP-G222E appeared to be folded similarly to fluorescent aceGFP and GFP, as indicated by pseudo-native PAGE and UV-light-induced photoconversion analyses. The finding of colourless acGFPL suggests that many non-fluorescent coelenterates may express similar invisible GFPs.

Photoconversion of aceGFP-G222E is a novel phenomenon, involving a UV light-induced transformation of immature protein into a blue-absorbing spectral form. A possible explanation for this is that maturation of the major part of the protein is terminated at a stage when cyclization of the protein backbone has occurred, but Tyr^{66} is still present in the native (non-dehydrogenated) form. UV irradiation drives dehydrogenation of the Tyr^{66} methylene bridge and formation of the complete chromophore with the conjugated system of π-electrons. Probably, analogous to *A. victoria* GFP, Glu^{222} is decarboxylated during this photoconversion, as suggested by the appearance of an absorption/excitation peak at 480 nm that corresponds to the deprotonated form of the chromophore.

aceGFP-G222E photoconversion appears to differ from GFP photoconversion under UV or blue light [19–21]. GFP chomophore conversion from a neutral (absorption at 390 nm) into a negatively charged (absorption at 475 nm) form occurs together with decarboxylation of Glu^{222}. Recently, a useful GFP mutant that can be used as a photo-activated label was generated [22]. Possibly, our mutant aceGFP-G222E can be used in a similar way, since fluorescence contrast between basal and UV-irradiated forms of this protein may exceed 1000-fold.

The role of Glu^{222} in acGFPL maturation appears intriguing, given the current understanding of GFP-like protein chemistry. Glu^{222} is invariant for all known GFP homologues and thus should play an important functional role. In GFP, Glu^{222} participates in proton transfer ensuring equilibrium between protonated and deprotonated forms of the chromophore [20]. Glu^{222} was proposed to catalyse chromophore formation [4,23]. In contrast, acGFPL Glu^{222} apparently inhibits formation of the complete chromophore.

The biological significance of acGFPL in jellyfish is unclear. This protein cannot act as a secondary emitter, as was postulated for *A. victoria* GFP. Thus acGFPL either lost any biological function during micro-evolution or plays some unknown role. Furthermore, spectral properties of *A. victoria* GFP allow us to surmise that its role might go beyond participation in bioluminescence. Indeed, the absorption spectrum of this protein with a major maximum at 396 nm and only a minor peak at 475 nm looks non-adapted to accepting energy from aequorin. From mutagenesis results we know that single mutations (e.g. S65T/G/C/A or E222G) readily convert GFP into a 470–490 nm-absorbing state [24,25] that is apparently much more suitable for absorption of aequorin emission. It might be expected that GFP would be easily converted into this state by natural selection if its only function was interaction with aequorin. Thus existence of GFP in its present spectral state suggests the necessity of the shorter-wavelength absorption peak for some still unknown process(es) in *A. victoria*.

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