Journal of Cellular Biochemistry Supplement 36:117–128 (2001)

Green Fluorescent Protein Variants Fold Differentially in Prokaryotic and Eukaryotic Cells

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Abstract Better-folding Green Fluorescent Protein (GFP) mutants selected from bacterial screenings are commonly used in widely different cellular environments. However, it is unclear if the folding efficiency of GFPs is invariant in different cell types. In this work, we have analysed the folding properties of GFP variants in bacteria versus mammalian cells. Remarkably, S65T was found to fold at comparable levels with the wild type GFP in bacteria, but at 10-fold lower levels in mammalian cells. On the other hand, Bex1 folded 3–4 times better than the wtGFP or S65T in E. coli, and 10–20-fold or more than 95-fold better, respectively, in mammalian cells. The Vex1 mutant demonstrated similar properties to Bex1. No evidence of differential GFP unfolding in vivo or of preferential degradation of unfolded GFP molecules was found. Moreover, no relationship between GFP folding efficiency and expression levels, or protein stability was detected. Trivial Aconfounding factors, like GFP unfolding caused by different pH or fluorescence quenching due to molecular crowding, were also excluded. In summary, our results demonstrate that specific GFP variants follow different folding trajectories in mammalian versus bacterial cells. The specificity of this differential folding supports a role of chaperones in guiding the folding of GFP in vivo. J. Cell. Biochem. Suppl. 36:117–128, 2001.

Key words: green fluorescent protein; protein stability; protein folding; gene mutation; molecular chaperones

The Green Fluorescent Protein (GFP) is a spontaneously fluorescent polypeptide that is widely used as a fluorescent tag for recombinant proteins [Chalfie et al., 1994; Cubitt et al., 1995; Prasher, 1995; Ludin and Matus, 1998]. GFP is physiologically expressed in the jellyfish A victoria [Chalfie et al., 1994], and expression at 37°C, a temperature dramatically higher than that of the habitat of Aequorea, profoundly reduces its folding efficiency [Ogawa et al., 1995; Heim and Tsien, 1996; Siemering et al., 1996; Patterson et al., 1997; Sacchetti and Alberti, 1999; Sacchetti et al., 2000]. Thus, numerous mutant GFPs have been generated, that are produced and folded more efficiently than the wild type (wt) molecule in bacteria [Heim et al., 1995; Anderson et al., 1996; Heim and Tsien, 1996; Kendall and Badminton, 1998; Sacchetti and Alberti, 1999; Sacchetti et al., 2000]. However, it is unclear if GFP molecules fold with the same efficiency in mammalian vs. bacterial cells. GFP is capable of auto-catalytic chromophore oxidation and folding [Heim et al., 1994; Kahn et al., 1997; Reid and Flynn, 1997; Sacchetti and Alberti, 1999]. However, cellular chaperones bind to GFP [Sakikawa et al., 1999; Weber-Ban et al., 1999], and can either increase its folding efficiency [Makino et al., 1997] or

Abbreviations used: orf, open reading frame; gfp, green fluorescent protein; qy, quantum yield; sb, streptavidin binding; wt, wild type.

Grant sponsor: Italian Association for Cancer Research (AIRC, Milano, Italy); Grant sponsor: Italian National Research Council (Convenzione CNR - Consorzio Mario Negri Sud, Rome, Italy); Grant sponsor: Italian Foundation for Cancer Research (FIRC) to A.S. and P.M.; Grant sponsor: Consortium for Tropical Medicine (CMT), Rome, Italy to T. El S.

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Received 18 October 2000; Accepted 5 December 2000

This article published online in Wiley InterScience, March 19, 2001.

cause unfolding [Weber-Ban et al., 1999]. A complex network of several different classes of chaperones exists within a cell [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000]. This structural diversity results in different mechanisms of catalysis [Agashe and Hartl, 2000] and in different specificity for protein substrates [Tian et al., 1995; Houry et al., 1999]. Major classes of cellular chaperones considerably diverge in eukaryotes compared with prokaryotes [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000]. As a consequence, protein-folding chaperones demonstrate significantly different mechanisms, protein specificity and kinetics in prokaryotic versus eukaryotic cells [Ellis, 1999; Feldman and Frydman, 2000; Panse et al., 2000a, b]. GFP molecules are thus expected to interact with different chaperones in different cell types, and this could critically affect the folding of GFP in vivo.

As a first test of this hypothesis, we have compared the folding efficiencies of wtGFP with first (S65T) [Heim et al., 1995] and second (Bex1 and Vex1) generation GFP mutants [Anderson et al., 1996] in bacteria and in mammalian cells. We demonstrate that, indeed, different GFP variants selectively fold with different efficiencies in mammalian cells vs. bacteria. These findings support an important role for cellular chaperones in GFP folding [Makino et al., 1997; Sakikawa et al., 1999; Weber-Ban et al., 1999], and suggest novel avenues to improve the folding efficiency of GFP in mammalian cells.

METHODS

Cells and Cell Culture

Human 293, 293T [DuBridge et al., 1987] and Hela cells, monkey COS-7 cells [Gluzman, 1981], and murine L cells were maintained in DMEM (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (Gibco-BRL).

GFP Expression in Mammalian Cells

The pRK-5 mammalian expression vector was a kind gift of Dr. J. Schlessinger and was used to express GFP as a soluble cytoplasmic protein [Sacchetti and Alberti, 1999]. The p10.1 plasmid contains the cDNA of the wtGFP [Chalfie et al., 1994], and was used as a PCR template. The S65T mutant was generated by directed mutagenesis of the wtGFP sequence. Bex1 and Vex1 were PCR amplified from pGL-2 constructs kindly supplied by Dr. M. Anderson [Anderson et al., 1996]. The pGFP-C1 (accession number: U19280) was obtained from Clontech (Palo Alto, CA). The following GFP variants were subcloned in pRK-5: (1) wtGFP−Δ5′UT, NcoI/EcoRI segment. This region of wtGFP contains a full length open reading frame (ORF) and the 3′UT region, but is devoid of the 5′UT region, (2) wtGFP, S65T [Heim et al., 1995], Bex1 [Anderson et al., 1996] and Vex1 [Anderson et al., 1996]. An N-terminal c-myc tag [Takaishi et al., 1995] was introduced by PCR using the following primers:

Forward primer: cgtctagaccatggaacaaaaactcatctcagaagaggatctgagtaaaggagaagaactt

Reverse primer: cggaagcttgggatatcacgcgtttgtatagttcatccat and, (3) wtGFP-SB. A C-terminal streptavidin binding (SB) tag [Sacchetti and Alberti, 1999] was introduced by PCR using the following primers:

Forward primer: gcgtctagacgcgtgatatcgcatatgagtaaaggagaagaa

Reverse primer: atggatgaactatacaaaagcgcttggcgtcacccgcagttcggtggttaaggatccaagcttgcg

Efficient transfection of mammalian cells was routinely achieved by calcium phosphate coprecipitation [Dell′Arciprete et al., 1996]. Cells were analysed 48 h after transfection. The tagged GFPs expressed in mammalian cells are indicated as wtGFP-myc, S65T-myc, Bex1-myc, Vex1-myc, or wtGFP-SB to distinguish them from the untagged wtGFP and wtGFP−Δ5′UT.

GFP Expression in Prokaryotes

The different GFP variants were subcloned from pRK-5 in the bacterial expression vector pET-29a or pET-11b (Novagen Inc., Madison, WI) [Studier et al., 1990] and expressed in MG-T7 cells [Studier et al., 1990]. The wtGFP(pBS) (pGFP, Clontech; accession number: U17997) was expressed in XL1-blue cells (Stratagene, La Jolla, CA).

GFP synthesis was induced with 0.3 mM IPTG (Calbiochem-Novabiochem, La Jolla, CA) in bacteria grown overnight as colonies on nitrocellulose filters or in exponential growth in liquid cultures.

GFP Protein Analysis

The amount of GFP protein (P) synthesized in bacteria or mammalian cells was quantified by Western blot analysis of cleared cell lysates. Bacterial lysates were obtained by sonication in PBS, 1 mM PMSF. Transfected mammalian

cells were lysed in PBS, 0.1% Triton, 2 mM PMSF. Western blots were performed as described previously [El-Sewedy et al., 1998], using rabbit anti-GFP antisera (Clontech). Hystidine-tagged GFP was purified under non-denaturing conditions using Ni-NTA agarose (Qiagen, Valencia, CA), and following the manufacturer's protocol. Protein concentrations were quantified with BCA (Pierce, Rockford, IL) or Biorad (Biorad, Munchen, Germany) assays, using BSA and RNAse as standards. The levels of GFP in the different samples were measured by densitometry, and analysed with NIH Image 1.62. A Kodak gray scale for standardization (http://www.kodak.com/country/US/en/motion/postProduction/tools/taf.shtml) was utilized to construct an exponential reference curve that was fed to NIH Image 1.62 to normalize the calculated values. Measurements of the Kodak gray samples optical densities were obtained on the electronic file, to prevent degradation of the measurement due to artifacts of film scanning and acquisition.

Absorption and Fluorescence Spectroscopy

Fluorescence measurements (F) on whole bacteria, cell lysates or purified GFP were obtained with a CM1T11I Spex spectrofluorimeter (Spex Industries, Edison, NJ). Measurements on whole bacteria were taken at a cell density of 0.2 OD_{600}. Absorbance spectra were measured with a Uvicon 860 spectrophotometer.

The GFP chromophore is a covalent double-ring structure, that is formed by condensation of the Ser65 and Gly67 in the wild type GFP [Cody et al., 1993; Tsien, 1998]. The cyclized chromophore is sufficient to absorb light, but requires the folding of the GFP β barrel to generate fluorescence [Tsien, 1998]. Correctly-folded, functional chromophores can be quantified by spectrofluorimetry, using correlated light absorption/fluorescence emission spectra. Cyclized chromophores with incorrect folding of the GFP β barrel can be measured by spectrophotometry (a good estimate is given by the absorbance at 448 nm upon alkali denaturation [Patterson et al., 1997]). The ratio between the spectrofluorimetric and spectrophotometric estimates indicates the fraction of functional chromophores in a GFP sample. An equivalent estimate is given by the ratio of the apparent versus real extinction coefficients of GFP samples purified to homogeneity. The apparent extinction coefficient (ε_a) was calculated as the ratio between the absorbance at optimal excitation wavelength and the GFP protein concentration. The real extinction coefficient (ε) of the same samples was calculated by normalization versus the 44100 $mol^{-1}cm^{-1}$ ε_{448} of NaOH-denatured GFP, used as an internal standard [Patterson et al., 1997; Sacchetti et al., 2000].

The Quantum Yield (QY) of the different GFP variants was calculated as previously described [Patterson et al., 1997], using fluorescein as a standard. The GFP P and F levels in mammalian cells were determined by Western blotting and spectrofluorimetry of cell lysates, respectively. The latter values were confirmed by flow cytometry (see below). Comparison between the mammalian F/P and the prokaryotic F/P allowed the determination of the fraction of correctly folded GFP in mammalian cells.

Fluorescence Microscopy

GFP-transfected cells were fixed in 4% paraformaldehyde (Sigma Chemical Company, St. Louis, MO) for 10 min at 4°C, and analysed on a Zeiss-Axiophot fluorescence microscope or a Meridian Insight Plus confocal microscope.

Flow Cytometry

Flow cytometric analysis was performed as previously described [Dell'Arciprete et al., 1996] with FACStar and Vantage flow cytometers (Becton Dickinson, Sunnyvale, CA) using conventional fluorescein optical settings. The $\varepsilon \cdot QY$ product is a measure of the efficiency of a chromophore [Matz et al., 1999]. In this respect, blue-excited and Vex1 GFPs are better chromophores than the wtGFP by 1.7- and 1.25-fold, respectively. However, the wtGFP is excited about 3.5-fold less at 488 nm than at 396 nm [Ward et al., 1982; Patterson et al., 1997]. Thus, blue-excited GFPs emit around 6-fold more light than the wtGFP, if both are excited at 488 nm. This coefficient was used to normalize the cytometric measurements of different GFP variants. Given the poor match of Vex1 excitation with the blue line of the argon ion laser, the Vex1 fluorescence was only quantified by spectrofluorimetry.

GFP Stability

Protein synthesis was blocked with cycloheximide (25 μg/ml) 40 h after transfection. At 16, 26, 36, and 44 h after the addition of cycloheximide cells were lysed with PBS, 0.1% Triton, 2

mM PMSF, and analysed for GFP protein and fluorescence levels.

RESULTS

Fluorescence Properties of GFP in Bacterial and Mammalian Cells

The spectra, QY and ε of the GFP variants under study, i.e. wild type, S65T (S65T), Bex1 (S65T, V163A), and Vex1 (V163A, S202F, T203Y) [Heim et al., 1995; Patterson et al., 1997; Sacchetti et al., 2000], were determined on the proteins expressed in bacteria and purified to homogeneity (Table I, data not shown). The spectra and ε of the GFP variants in mammalian cells were determined on cell lysates. No significant differences were detected between these parameters in the two cell types, whether measured on tagged or untagged GFP variants.

The cytoplasmic pH of mammalian 293 cells is around 7.3 [Schuhmann et al., 1997], whereas that of E. coli is between 7.4 and 7.6 [Conley et al., 1994]. The fluorescence properties and folding stability of GFP variants is differentially affected by pH [Ward et al., 1982; Patterson et al., 1997; Sacchetti et al., 2000]. Thus, the fluorescence spectra and stability of all the GFP variants under study were determined upon prolonged exposure to pH 5 to 10. wtGFP and Vex1 proved more stable than S65T and Bex1 at lower pH. However, the differences in spectral properties and chromophore stability between pH 7 and 8 proved negligible (data not shown), and did not significantly affect the comparison between GFP in bacteria vs. mammalian cells.

Different concentrations of GFP in mammalian versus bacterial cells might lead to a differential fluorescence quenching, and affect comparisons artefactually. Under the experimental conditions described above, about 4×10^8 molecules of wtGFP and S65T are produced per bacterial cell at 37°C. Bex1 and Vex1 reach 4–5-fold higher steady-state levels than the wtGFP (Table I). Western blot analysis indicates that 293T cells produce about 7×10^9 wtGFP-myc molecules per cell. Bex1-myc and S65T-myc transfected cells synthesize 2×10^9 and 6×10^9 GFP molecules per cell, respectively. Assuming an average volume of 2 fl for bacteria and of 100 fl for mammalian cells, cytoplasmic GFP molecules can reach the considerable concentration of 0.7–3.0 M in prokaryotic cells (37°C), and of 60–200 mM in mammalian cells. Thus, undiluted and serially-diluted GFP-containing lysates of bacteria induced at room temperature (several times more concentrated than the above) were analysed spectrofluorimetrically for the possible occurrence of fluorescence quenching. No evidence for the latter was found. However, an inner-filter effect [Scholz et al., 2000] due to the abundance of GFP and to the relatively long optical path of the cuvette (2 mm) was observed for both wtGFP and Bex1-GFP lysates. This

TABLE I. Efficiency of Expression of GFP Variants in Bacteria

GFP*	F[e]	P[a]	ε[b]	ε_a[c]	Qy[d]	Folded molecules[e] ($\varepsilon_a/\varepsilon$)
wtGFP						
37°C	1[f]	1[f]	28000 ± 1300	5600 ± 2500	0.8	20 ± 9
RT	75	20	–	20100 ± 3000		72 ± 11
S65T (S65T)						
37°C	0.9 (1.5)	1.1	63600 ± 2900	9950 ± 4700	0.6	16 ± 7
RT	25 (42)	6	–	40400 ± 5000		63 ± 8
Bex1 (S65T, V163A)						
37°C	21 (36)	5	63700 ± 1800	40700 ± 6600	0.6	65 ± 10
RT	159 (270)	28	–	59400 ± 6100		93 ± 10
Vex1 (V163A, S202F, T203Y)						
37°C	24 (30)	4	35100 ± 600	34300 ± 1700	0.8	98 ± 5
RT	65 (81)	12	–	36600 ± 4700		104 ± 13

*GFP variants; mutations are in brackets
[e]GFP fluorescence emission by spectrofluorimetry (at optimal excitation wavelength [Sacchetti et al., 2000]: $wtGFP_{396}$, $S65T_{489}$, $Bex1_{489}$, $Vex1_{398}$). To permit direct comparisons, the values in the table are normalize versus wtGFP and divided by the optical correction coefficient; undivided values are indicated in brackets
[a]Relative amounts of GFP per cell
[b]Real GFP extinction coefficients (mean ± SD)
[c]Apparent GFP extinction coefficients
[d]Quantum yield
[e]Ratio between apparent and real extinction coefficients, expressed as percentage
[f]Reference value for normalization

caused a reduction in the fluorescence emission of the undiluted lysates of 45% and 43%, respectively. A 1/8 dilution of the lysates was sufficient to eliminate this effect, and to revert to a linear correlation between GFP concentration and signal intensity. Due to the small diameter of the cells analysed and to the lower amounts of GFP produced at 37°C (Tables I and II), this inner-filter effect is unlikely to be significant in vivo. Still, for safety of comparison, fluorimetric measurements on whole cells (flow cytometry and confocal microscopy) were taken in parallel to a spectrofluorimetric analysis of diluted cell lysates. The two methods produced comparable quantitative estimates of fluorescence emission.

Taken together, these results indicate that the GFP fluorescence properties and assay conditions are analogous in bacteria and mammalian cells, and permit a reliable comparison of the GFP folding in the two cell types.

GFP Expression in Bacteria

GFP synthesis was induced with 0.3 mM IPTG at 37°C in bacteria grown overnight as colonies on nitrocellulose filters or in exponential growth in liquid cultures. The expression levels of wtGFP, S65T, Bex1, and Vex1 in bacteria grown as colonies did not significantly differ. However, Bex1 and Vex1 reached 5- and 4-fold higher steady state levels, respectively, than wtGFP and S65T in bacteria in exponential growth in highly oxygenated liquid cultures. Exponential growth in medium allowed for a more efficient production of the different GFP variants and was used for all subsequent comparisons.

The production of the different GFP variants was differentially affected by the induction time [Heim et al., 1995]. Bex1, Vex1, and S65T reached maximal protein expression and fluorescence levels faster than the wtGFP in pET [Heim et al., 1995]. The wtGFP in pBS (pGFP) was induced even more slowly. However, both pET-wtGFP and pBS-wtGFP reached comparably high expression levels after overnight induction. Overnight induction was chosen to allow for the highest expression levels of all GFP variants under study.

The fraction of soluble GFP protein vs. aggregates was analysed at different time points (from 5 min to 20 h) upon induction with IPTG. The highest producers generated the largest fraction of insoluble GFP at all time points, consistent with earlier reports [Heim and Tsien, 1996; Tsien, 1998]. At mid-time points, the soluble fraction of Bex1 and Vex1-GFP was only 20–30%, whereas that of the wtGFP was 30–40% and that of S65T-GFP was 50%. Since GFP aggregates are largely unfolded [Reid and Flynn, 1997], both bacterial and mammalian lysates were centrifuged before analysis, and soluble GFP material was analysed for chromophore folding.

TABLE II. Efficiency of Production of GFP in Mammalian Cells

GFP form	F*	P[a]	F/P[b]	Folded molecules[c]
293T cells				
wtGFP	0.05	0.01	5.0	25.0
wtGFP–Δ5′UT	8.0	1.7	4.7	23.5
wtGFP (pGFP-C1)	0.26	0.05	5.2	26.0
wtGFP-myc[d]	1	1	1	5.0
S65T-myc	0.1 (0.17)	0.8	0.1	0.5
Bex1-myc	5.7 (9.7)	0.6	9.5	47.5
Vex1-myc	4.6 (5.75)	0.4	11.5	57.5
wtGFP-SB	3.7	0.3	12.3	61.5
COS-7 cells				
wtGFP–Δ5′UT	8.0	1.1	8.8	22.0
wtGFP-myc[e]	1	1	1	2.5
S65T-myc	≤ 0.1 (≤ 0.17)	0.6	≤ 0.2	≤ 0.5
Bex1-myc	7.8 (13.3)	0.4	19.5	48.5
Vex1-myc	6.3 (7.9)	0.2	31.5	79.0
wtGFP-SB	2.8	0.1	28.0	70.0

*GFP fluorescence emission by spectrofluorimetry (excitation at optimal absorption wavelength). To permit direct comparison, the values in the table are normalized vs. wtGFP and divided by the optical correction coefficient; undivided values are indicated in brackets

[a]GFP protein expression levels were estimated by Western blot

[b]Ratios between GFP fluorescence and protein levels

[c]The fraction of functional chromophores was calculated as in Material and Methods, and expressed as percentages

[d]Reference values for normalization in 293T cells

[e]Reference values for normalization in COS-7 cells

Folding Efficiency of GFP in Bacteria

GFPs produced at 37°C were purified to homogeneity and analysed for fluorescence properties and folding levels (Table I). To quantify the optimal folding of each GFP variant, these were also produced at room temperature. Mutations internal to the GFP ORF heavily affected the GFP folding efficiency (Table I). Only 16% of the S65T molecules synthesised at 37°C were fluorescent (Fig. 2A and Table I), whereas Bex1 and Vex1 were 65% and 98% functional, respectively (Fig. 2B and Table I). All the GFP variants folded more efficiently at RT than at 37°C, confirming that the GFP structure is destabilized at higher temperatures (Table I). Notably, the wtGFP showed an almost 4-fold increase of the folded fraction at room temperature, and a 75-fold increase of the overall level of fluorescent GFP molecules per cell, confirming that the wtGFP is physiologically optimized for expression at low temperatures, and that the level of folding is a limiting factor in GFP expression at 37°C.

Stability of GFP Folding in Bacteria

The fluorescence spectra and F/P ratios of GFP purified from bacteria induced for short (15 and 60 min) or long (5 and 20 h) times were determined. The folding of GFP proved to be considerably fast [Siemering et al., 1996], and the percentages of functional chromophores at 15 min were essentially identical to those of later time points. The GFP absorption and emission spectra and F/P ratios also proved essentially invariant at later time points, indicating that very little, if any, unfolding [Weber-Ban et al., 1999; Panse et al., 2000a] of GFP occurs in bacteria. Consistently, no absorption peaks of denatured GFP [Cody et al., 1993] were detected at any time point (data not shown).

GFP Expression in Mammalian Cells

The various GFP constructs (wtGFP, wtGFP $-\Delta 5'$UT, pGFP-C1, wtGFP-myc, S65T-myc, Bex1-myc, Vex1-myc, and wtGFP-SB) were expressed in different mammalian cell lines (293T, 293, COS-7, HeLa, and L cells). Analysis of transfected cells by conventional and confocal fluorescence microscopy demonstrated that the GFP constructs were efficiently expressed in the transfected cells (Fig. 3). Notably, wtGFP-myc in pRK-5 is expressed at 20-fold higher levels than pGFP-C1 [Cheng et al., 1996; Crameri et al., 1996] in 293T cells. pRK-5 and pGFP-C1 bear different viral promoters (RSV versus CMV, respectively). The pGFP-C1 also carries an S2G mutation, that appears detrimental to GFP expression (unpublished data). Since pGFP-C1 has been routinely used as a wtGFP reference standard [Cheng et al., 1996; Crameri et al., 1996], some of the reported improvements in performance of GFP mutants over the wtGFP in eukaryotic cells have been overestimated.

The average GFP protein levels varied in the different cell lines (293T > 293 > COS-7 >> HeLa = L cells) (Table II, Figs. 2B, 4 and data not shown). However, the relative expression levels of the GFP mutants appeared invariant in the different cell lines. The expression efficiency of the different GFP variants was analysed in detail in transiently-transfected 293T and COS-7 cells (Table II, Figs. 2B, 4). The absolute GFP protein expression levels in 293T cells were 3.5- to 5-fold higher than in COS-7 cells. However, no differences in the relative expression levels of different GFP variants were observed in the two cell lines (Table II, and Fig. 2B). Analogously, 293 cells invariably expressed all the different GFP variants at about 2-fold lower levels than 293T cells (data not shown).

All the mutant GFPs were expressed at lower steady-state protein levels than the wild type in mammalian cells (Table II, Fig. 2B). Notably, Bex1-myc and Vex1-myc only reached 50% of the wtGFP-myc protein level in mammalian cells, whereas the corresponding constructs were expressed at 5-fold higher levels than wtGFP in bacteria (Tables I, II, and Fig. 2A,B). A differential protein stability, together with a variable translation ability of the corresponding mRNA, accounts for these results (manuscript in preparation).

Folding Efficiency of GFP in Mammalian Cells

The folding levels of the GFP variants analysed (Table II, Figs. 2, 4) were calculated as in Methods. Notably, the folding efficiency of each GFP variant proved essentially invariant across different mammalian cell lines (Table II).

GFP protein tags and ORF mutations (Fig. 1) heavily affected the efficiency of folding in mammalian cells. Remarkably, the ORF mutations of S65T, Bex1 and Vex1 were found to affect the folding of GFP in mammalian cells differently than in bacteria (Table I vs. II).

S65T-myc demonstrated a much poorer performance than the wtGFP-myc, and produced 10-fold fewer functional chromophores. On the other hand, Bex1-myc showed a 95-fold better folding efficiency than S65T-myc, demonstrating that the V163A mutation is sufficient to improve the GFP folding efficiency in mammalian cells [Crameri et al., 1996; Patterson et al., 1997]. This also demonstrates that the presence of the myc-tag, albeit diminishing the fraction of folded GFP molecules [Waldo et al., 1999], does not necessarily impede higher folding levels. The even higher folding demonstrated by the other V163A mutant analysed (Vex1-myc) confirmed these findings.

A C-terminal streptavidin-binding tag (Fig. 1) dramatically increases the folding of wtGFP in mammalian cells [Sacchetti and Alberti, 1999], inducing a 3-fold better folding than the other wtGFP constructs. This increase in folding (more than 60% of the molecules were correctly folded) was seen both in 293T and COS-7 cells, indicating that these results may have widespread validity in mammalian cells. Comparison of wtGFP-SB with wtGFP-myc confirmed recent findings that polypeptides fused to GFP dictate markedly different folding levels of otherwise identical molecules [Sacchetti and Alberti, 1999; Waldo et al., 1999]. These results open the possibility of tuning the GFP folding to different levels, and of using GFP mutants to study fundamental characteristics of protein folding, e.g. intramolecular chaperones [Shinde et al., 1999] or folding-antagonist sequences [Waldo et al., 1999]. Moreover, they confirm that the experimental conditions used in this work are permissive for high GFP folding levels, and are not a limiting factor in the comparison of the different mutants under study.

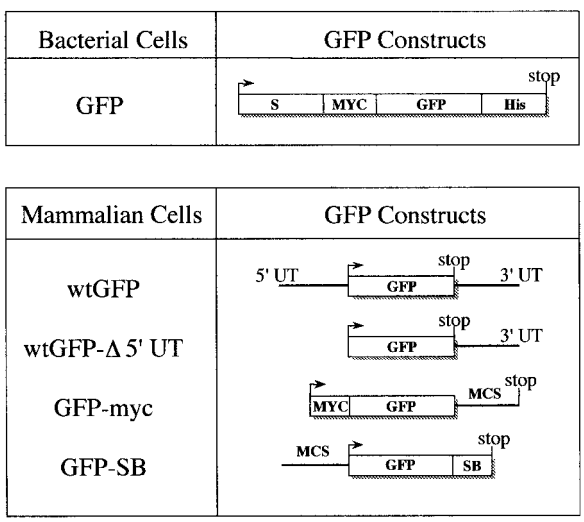

Fig. 1. GFP expression constructs for bacterial and mammalian cells. (top) pET-29a constructs (wtGFP, S65T, Vex1 and Bex1) for expression in E. coli. S: S tag; MYC: myc tag; His: 6-histidine tag. wtGFP was also expressed with an SB-tag in pET-11b. (bottom) pRK-5 constructs for expression of GFP in mammalian cells. wtGFP: full length cDNA; wtGFP−Δ5′UT: wtGFP devoid of the 5′UT; GFP-myc: wtGFP, S65T, Vex1 or Bex1 with a myc tag; GFP-SB: wtGFP with SB-tag. UT: untranslated region; bent arrow: Met initiation of translation codon; MCS: multiple cloning sites; STOP: stop codon.

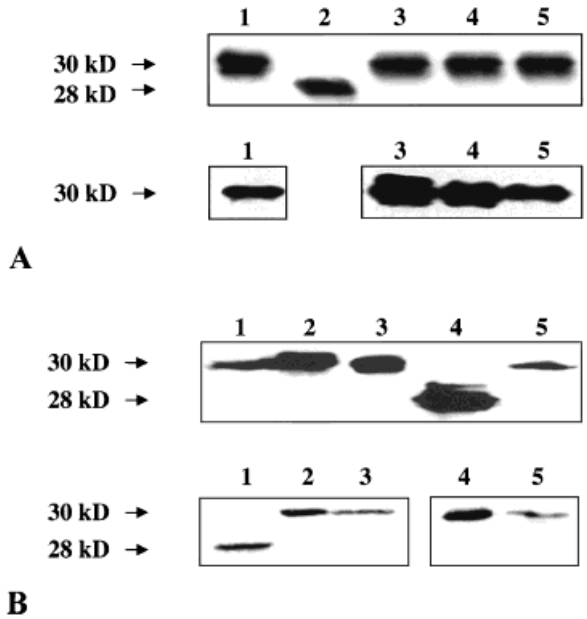

Fig. 2. Western blot analysis of GFP production. **(A)**: E. coli. (top) Bacterial colonies grown on filter; (bottom) Bacteria grown in liquid culture. (1) wtGFP; (2) pGFP (wtGFP in pBS); (3) Bex1; (3) Vex1; (4) S65T. **(B)**: Mammalian cells. (top) 293T cells; (bottom) COS-7 cells. (1) Bex1-myc; (2) wtGFP-myc; (3) S65T-myc; (4) wtGFP−Δ5′UT; (5) Vex1-myc. Lanes 1–3 and 4–5 of the bottom panel are from two independent experiments.

Remarkably, the folding efficiency of the GFP variants analysed proved independent of their absolute levels of expression. A clear example is that of the wtGFP constructs. The wtGFP, wtGFP−Δ5′UT, and wtGFP (pGFP-C1) in 293T cells, and the wtGFP−Δ5′UT in COS-7 cells are expressed at widely different levels, largely depending on the presence or absence of the GFP 5′UT [Chalfie et al., 1994] (Table II). Never the less, they demonstrate a similar 20–25% folding efficiency. This corresponds well to the folding of the wtGFP in bacteria at 37°C, indicating that this is a characteristic folding level of wtGFP at this temperature.

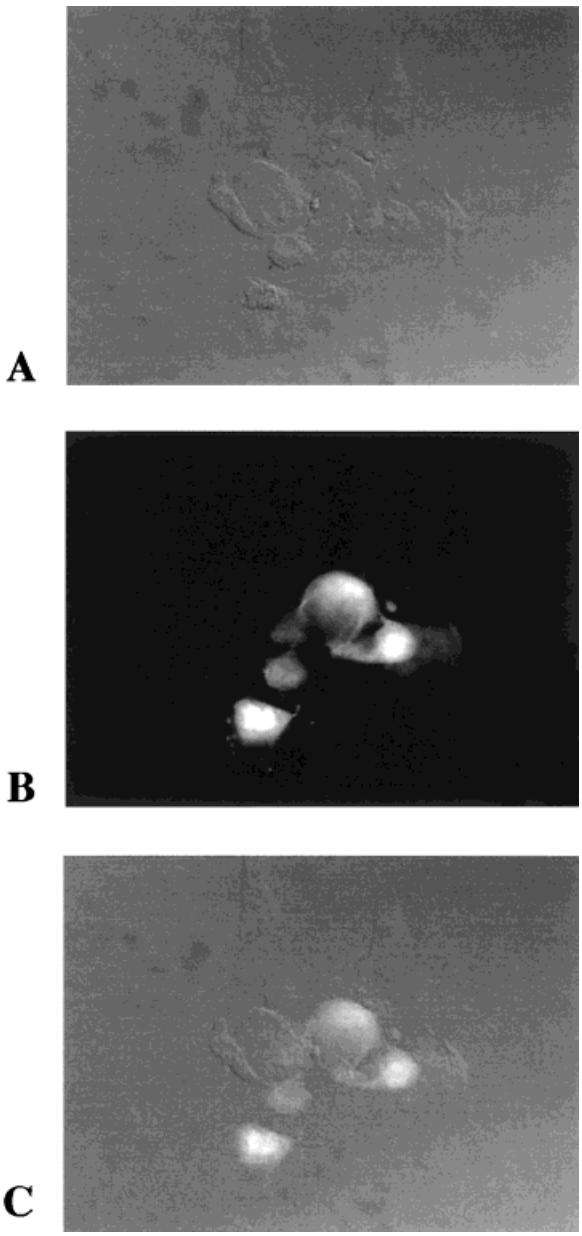

Fig. 3. Optical microscopy analysis of GFP expression in mammalian cells. wtGFP-myc-transfected 293T cells were analysed by: **(A)**: Interference optics **(B)**: UV excitation and fluorescence collection **(C):** Combination of interference optics and fluorescence collection.

Stability of the GFP Protein and of its Folded State in Mammalian Cells

The results presented above raised two major issues. The first one was if the added peptidic tags or GFP mutations affected the folding levels by altering the protein stability in the cells, for example through a preferential degradation of unfolded molecules. A second issue was if significant GFP unfolding occurred in vivo [Weber-Ban et al., 1999; Panse et al., 2000a].

If GFP folding affects GFP stability, high or low GFP folding levels would be expected to correlate with slow or fast protein degradation, respectively. The GFP protein degradation curves were determined by Western blot analysis at different times after a blockade of protein synthesis with cycloheximide (Fig. 5B, and manuscript in preparation). The greatest stability was shown by wtGFP, that demonstrates a half-life of 54 h. The addition of protein tags or the introduction of mutations in the GFP ORF considerably decrease the stability of GFP (Fig. 5A, see wtGFP vs. wtGFP-SB or -myc; see also wtGFP-SB or -myc vs. S65T-myc, Bex1-myc and Vex1-myc).

However, no correlation was found between the stability of the GFP variants and their folding levels. Indeed, wtGFP is much more stable than Bex1-myc, Vex1-myc, and wtGFP-SB, GFP variants that demonstrate 2–3-fold higher folding levels than the wild type (Fig. 5A,B). Analogously, the decay curve of wtGFP-myc essentially coincides with that of Bex1-myc, and that of Vex1-myc largely overlaps with that of S65T-myc, in spite of large differences in their respective protein folding levels. Taken together, these results indicate that the folding efficiency of GFP is not significantly affected by GFP stability. They also indicate that the V163A mutation has no beneficial role on GFP protein stability.

If the GFP unfolding rate was significant, a faster decay of the fluorescence levels compared with the protein levels would have to be expected. Quite to the contrary, the fluorescence decay curves were demonstrated to closely mirror their respective protein decay profiles (Fig. 5B). This indicated that very little, if any, GFP unfolding occurs in vivo. This is consistent with previous findings that GFP resists unfolding caused by several physical and biological agents, i.e. heating (65°C), pH extremes, chaotropic agents (6 M guanidine) and proteases [Cubitt et al., 1995].

DISCUSSION

GFP is a proteic chromophore from the jellyfish A. victoria [Chalfie et al., 1994]. Expression at 37°C, a temperature dramatically higher than that of the habitat of Aequorea, profoundly

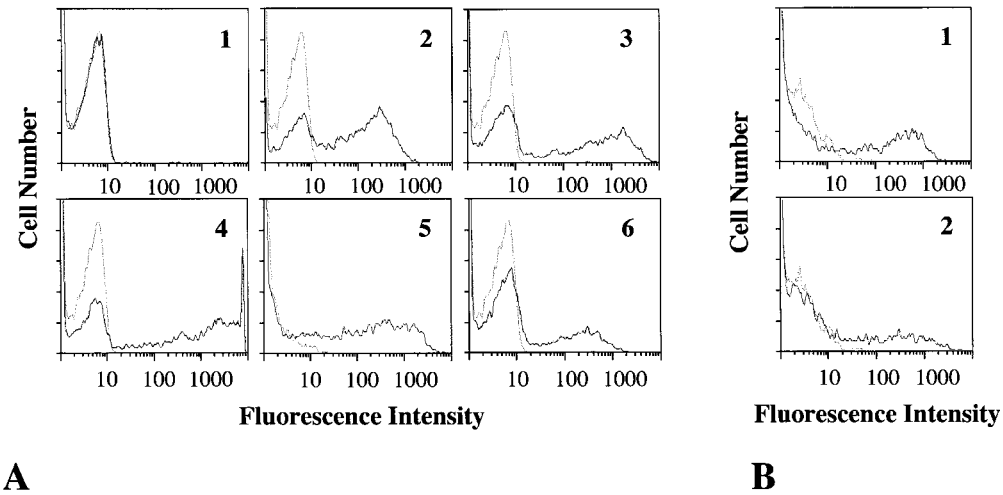

Fig. 4. Flow cytometric analysis of GFP expression in mammalian cells. **(A)**: 293T cells. The cells were transfected with (solid line): (1) wtGFP; (2) wtGFP-myc; (3) Bex1-myc; (4) wtGFP−Δ5′UT; (5) wtGFP-SB; (6) S65T-myc. Dotted line: vector alone-transfected cells. **(B)**: COS-7 cells. The cells were transfected with (solid line): (1) wtGFP-myc; (2) wtGFP-SB. Dotted line: vector alone-transfected cells.

reduces the folding efficiency of GFP [Heim and Tsien, 1996; Siemering et al., 1996; Sacchetti and Alberti, 1999; Sacchetti et al., 2000]. Thus, numerous GFP mutants have been generated that fold more efficiently than the wild type at 37°C in bacteria [Heim and Tsien, 1996; Sacchetti et al., 2000]. However, it is unclear if a similar efficiency is maintained in eukaryotic cells. The results presented in this article demonstrate that this is not the case, and suggest a major role of cellular chaperones in GFP folding.

To determine if the process of GFP folding differs in mammalian vs. bacterial cells, the synthesis, fluorescence, stability, and folding properties of different GFP variants were determined in the two cell types. The GFP variants were chosen so as to have a representative, yet sufficiently manageable, spectrum of mutants with different fluorescent characteristics and different numbers of mutations. Our results show that wtGFP folds at comparable levels in E. coli and in mammalian cells. On the other hand, S65T folds similarly to wtGFP in E. coli, but demonstrates 10-fold lower folding levels than wtGFP in mammalian cells. The V163A mutant Bex1 folds 3–4-times better than wtGFP or S65T in E. coli, at 37°C, but

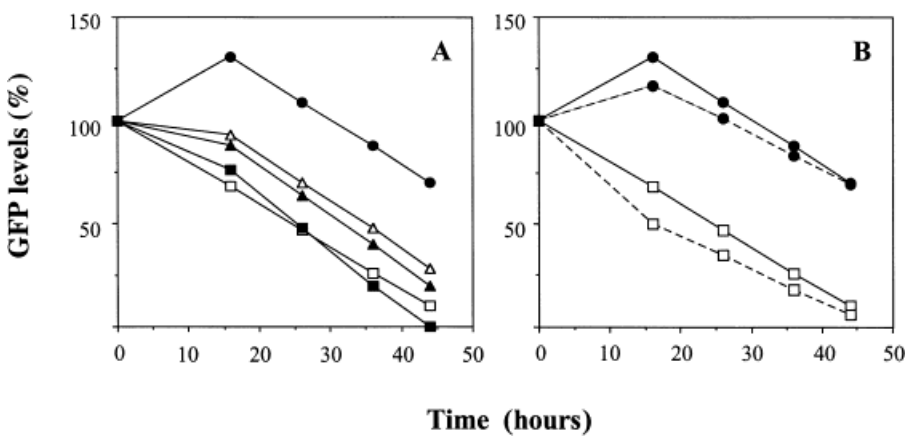

Fig. 5. GFP stability in transiently transfected 293T cells. **(A)**: Decay rates of GFP fluorescence: wtGFP−Δ5′UT (•); wtGFP essentially overlapped with wtGFP−Δ5′UT; wtGFP-myc (△); GFP-SB essentially overlapped with wtGFP-myc; Bex1-myc (▲); S65T-myc (closed squares); Vex1-myc (□). The increased levels of wtGFP at short times after the cycloheximide treatment suggest a faster decay of GFP-degrading enzymes. **(B)**: Protein and fluorescence decay rates of wtGFP−Δ5′UT (closed circles) and Vex1-myc (□). Solid line: fluorescence levels; dashed line: protein levels.

folds 10–20-times, and more than 95-times better than these variants, respectively, in mammalian cells. The Vex1 mutant demonstrates similar folding characteristics to Bex1, confirming the more efficient folding of V163A mutants in mammalian cells [Anderson et al., 1996]. These findings and the high folding levels of the SB-tagged GFP demonstrate that mammalian cells do not impede an efficient GFP folding. This implies that the poor performance of some GFP variants in mammalian cells is characteristic of their structure. The divergent folding efficiencies of GFP variants were consistently found in different mammalian cell lines, thus suggesting the widespread validity of this finding.

A differential degradation of unfolded vs. folded GFP molecules could have significantly affected the steady-state levels of folded GFP. In particular, a preferential degradation of unfolded molecules would be expected to result in a shorter half-life of poorly-folding GFP variants. Since this could have biased estimates of the folding ability of different GFPs, the GFP fluorescence and protein decay rates were determined after a protein synthesis blockade. wtGFP demonstrated a remarkably long half-life of 54 hours. All the GFP variants analyzed proved less stable than wtGFP in mammalian cells, indicating that bacteria-selected mutants or tagged versions of GFP are not optimized for high protein stability in mammalian cells. N-terminal residues (myc-tag) can affect protein degradation rates [Varshavsky, 1997, Dantuma et al., 2000]. However, C-terminal tags or GFP ORF mutations similarly reduce the GFP half-life, indicating that additional factors, e.g. an altered GFP structure [Ormo et al., 1996; Wachter et al., 1997], induce a faster degradation of GFP. A comparison between S65T and Bex1 demonstrates that the introduction of a V163A mutation considerably increases the GFP protein stability in eukaryotic cells. However, with the second V163A mutant, Vex1 demonstrates a much shorter half-life. Thus the effect of the V163A mutation on protein stability depends on the sequence in which it is expressed. It should be noted that the V163 is internal to the β-barrel. Comparative analysis of the V163A mutant 3D-structures versus the native V163 indicate that the modest decrease in the volume occupancy of this side chain is sufficient to subtly alter the structure of the GFP β-barrel and the orientation of several side chains in the outside surface of GFP (unpublished observations). Peptide tags heavily affected the folding of GFP. In particular, the addition of a streptavidin-binding tag induced an impressive 3-fold better folding of wild type GFP. However, wtGFP-SB did not demonstrate a higher protein stability than wtGFP. In summary, the folding efficiency does not dictate the stability of the GFP protein in mammalian cells, and, conversely, a differential protein stability cannot account for the observed differences in the folding of GFP variants.

The occurrence of significant GFP unfolding in vivo [Weber-Ban et al., 1999; Panse et al., 2000a] could have differentially affected the steady-state levels of folded GFP variants and biased estimates of true folding capability. A faster decay of fluorescence vs. protein levels was expected if significant GFP unfolding occurs in vivo. However, the decay curves of the GFP peptides corresponded well to those of the active chromophores for all the GFP variants analyzed (Fig. 5B and data not shown). This indicates that very little, if any, GFP unfolding occurs in vivo, and that it cannot significantly affect the steady-state levels of folded GFP.

The absolute expression levels of the GFP variants did not affect their folding efficiency. A good example is that of wtGFP–Δ5′UT versus wtGFP. The two proteins are identical, but are produced at 200-fold different levels in mammalian cells, due to the inhibitory effect of the 5′UT of the gene on GFP expression [Chalfie et al., 1994]. Nevertheless, the proteins originating from the two constructs demonstrate the same folding efficiency. Analogously, different GFP variants demonstrate considerably different expression levels in different cell lines, but reach the same folding levels.

Taken together, our results demonstrate that GFP variants fold differentially in mammalian cells compared to bacteria. GFP is capable of auto-catalytic chromophore oxidation and folding [Heim et al., 1994; Kahn et al., 1997; Reid and Flynn, 1997; Sacchetti and Alberti, 1999]. However, it is difficult to imagine how GFP molecules could differentially change their folding trajectories in bacteria vs. mammalian cells. Differences in the molecular apparatus that actively guides GFP folding could, on the other hand, explain these findings well. Cellular chaperones have been demonstrated to bind GFP [Sakikawa et al., 1999; Weber-Ban et al., 1999] and can induce folding [Makino et al.,

1997] or cause unfolding [Weber-Ban et al., 1999] of GFP. Widely diverse structural classes of intracellular folding chaperones exist in a cell [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000], and result in different mecanisms of catalysis [Agashe and Hartl, 2000], thermodynamics [Panse et al., 2000a, b], and substrate specificity [Tian et al., 1995, Houry et al., 1999]. This diversity is even more striking when comparing chaperones in bacteria and mammalian cells [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000]. As a consequence, GFP molecules are expected to interact with chaperones possessing dramatically different structures, folding mechanisms and peptide sequence specificity [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000] in bacteria vs. mammalian cells.

Not only N- or C-terminal peptidic tags affect GFP folding, but also mutations of internal residues, e.g. S65, that might not be expected to directly interact with GFP chaperones. However, significant protein folding occurs cotranslationally [Fedorov and Baldwin, 1997]. Moreover, folding chaperones induce recursive cycles of folding and unfolding of their protein targets [Martin and Hartl, 1997; Ruddon and Bedows, 1997; Feldman and Frydman, 2000]. Thus, significant exposure of internal GFP residues to chaperones can occur during the folding process. Interestingly, this might help explaining discrepancies between the important consequences of some mutations on protein folding and the apparent irrelevancy of their location versus the overall structure of GFP [Cubitt et al., 1995]. Alternatively, the analysis of the GFP structure [Ormo et al., 1996; Wachter et al., 1997; Perozzo et al., 1998; Prendergast, 1999] demonstrates that significant long-range alterations of the GFP external conformation can be induced by single, internal, aminoacid changes. Thus, mutation of non-exposed residues could even affect the binding of chaperones to intact GFP molecules.

Taken together, these findings support an important role of cellular chaperones in GFP folding [Makino et al., 1997; Weber-Ban et al., 1999]. The use of GFP mutants may help to shed light on fundamental mechanisms of protein folding. Novel avenues to improve the folding efficiency of GFP in mammalian cells might also be designed, and screening procedures based on mammalian cells are indicated for further GFP optimization.

ACKNOWLEDGMENTS

We thank Dr. J. Schlessinger for material and support. We also thank Dr. M. G. Capri and F. Lorenzini for help, and Dr C. P. Berrie for critical appraisal of the manuscript.

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