pH-Dependent Fluorescence of a Heterologously Expressed *Aequorea* Green Fluorescent Protein Mutant: In Situ Spectral Characteristics and Applicability to Intracellular pH Estimation[†,‡]

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ABSTRACT: The green fluorescent protein of *Aequorea victoria* (GFP) is a natural peptide chromophore without substrate or cofactor requirements for fluorescence. In vitro, a recombinant F64L/S65T GFP mutant (GFPmut1) exhibited pH sensitive fluorescence within the physiologic range. When heterologously expressed in BS-C-1 cells or rabbit proximal tubule cells, uniform cytosolic and nuclear fluorescence was observed. Cytosolic fluorescence constituted over 80% of the total. Excitation scanning of transfected cells revealed two GFPmut1-specific regions that were pH-sensitive over the physiologic range, and each region exhibited a unique pH "bias" in fluorescence emission. Excitation at or near the expected maximum of 488 nm (region II) uniformly resulted in fluorescence that was preferentially altered at acidic pH. In contrast, a novel "wild-type" excitation peak at 400 nm (region I) resulted in alkaline-biased fluorescence similar to that described for the wild-type chromophore in vitro, suggesting that wild-type spectral features disrupted in vitro by mutagenesis may be recovered in intact cells. Calibration of intracellular pH (pH_i) with in situ fluorescence following excitation in either region revealed a semilogarithmic relationship between fluorescence intensity and pH within the physiologic range. We therefore measured pH_i changes attributable to altered Na/HCO_3 cotransport (NBC) activity both in GFPmut1-expressing cells and in paired untransfected cells loaded with BCECF. Basal NBC activity was the same in each group, as was the stimulation of activity by 10% CO_2, thus validating the utility of GFPmut1 as a fluorescent probe for pH_i and establishing a novel, useful, and practical application for GFPmut1 in monitoring pH_i in real time.

Cnidarian green fluorescent proteins (GFPs)[1] are small, naturally occurring bioluminescent proteins that primarily fluoresce in response to radiant energy transfer and therefore differ from luciferases in that they do not require either substrates or cofactors for fluorescence. Following the cloning of the *gfp10* cDNA (*1*) which encodes the GFP of the jellyfish *Aequorea victoria*, functional GFPs have been heterologously expressed in cell types as phylogenetically diverse as bacteria, plants, and mammalian cells (*2*−*4*). This has led to a rapid sucession of novel and useful molecular biology applications for both wild-type and mutant GFPs [for review, see (*5*)]. GFPs have been used as reporters of gene expression (*2*, *6*−*8*), as markers of cell lineage (*5*), and as peptide fusion tags to monitor both intracellular peptide localization (*9*−*11*) and protein trafficking (*12*−*14*). Their small size [∼27 kDa (*15*)], nonperturbing nature, and remarkable stability under a variety of denaturing conditions (*16*)—coupled with their ability to retain their intrinsic fluorescence when fused to other peptides—has made them an indispensable part of the molecular biology armamentarium. In fact, their utility has been limited only by low sensitivity and delayed expression. Recently, several red-shifted mutants have been described with both enhanced fluorescence and more rapid expression relative to wild-type GFP, thereby dramatically increasing the usefulness and sensitivity of studies employing these bioluminescent molecules.

The in vitro spectral characteristics of both purified and recombinant wild-type GFPs are influenced by ambient pH (*16*−*19*). Similar pH sensitivity has recently been reported for a number of other GFP variants (*20*), suggesting pH

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[1] Abbreviations: GFP, green fluorescent protein; pH_i, intracellular pH; NBC, Na/HCO_3 cotransporter; RPTC, rabbit proximal tubule cells; BCECF, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; BCECF-AM, triacetoxymethyl ester of BCECF; FITC, fluorescein isothiocyanate; PMT, photomultiplier tube; PBS, phosphate-buffered saline; FBS, fetal bovine serum; PMSF, phenylmethanesulfonyl fluoride; F, phenylalanine; S, serine; L, leucine; T, threonine.

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sensitivity for this entire class of proteins. The underlying mechanism of pH-dependent changes in fluorescence is thought to involve altered protonation of the fluorogenic core of the molecule (*16*, *18*, *19*, *21*−*23*). These changes are both rapid and readily reversible for wild-type GFP in vitro, even at pH extremes (*16*). pH-sensitive spectral changes in the physiologic range, however, have received little attention, and the influence of pH on the spectral characteristics of GFPs in living cells has not previously been addressed.

The red-shifted GFP variant, GFPmut1, contains a double amino acid substitution (F64L/S65T) that results in basal fluorescence intensity 35-fold greater than its wild-type precursor (*24*). In preliminary studies of renal epithelial cells heterologously expressing GFPmut1, we observed reversible changes in fluorescence under conditions known to alter intracellular pH (pH_i). These observations—made in both cultured rabbit proximal tubule cells and BS-C-1 cells, a continuous epithelial-like cell line of African Green Monkey kidney origin (*25*, *26*)—suggested that GFPmut1 fluorescence was pH-sensitive in intact cells. We therefore sought to characterize the spectral features of recombinant GFPmut1 in situ and to test whether this important class of bioluminescent proteins could be employed to probe pH_i.

MATERIALS AND METHODS

Chemical Reagents. 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was obtained from Molecular Probes (Eugene, OR) as its caged triacetoxymethyl ester (BCECF-AM) and was prepared and stored according to the manufacturer's recommendations. All cell culture medium and additives (e.g., G418 sulfate) were obtained from GibcoBRL (Gaithersburg, MD). All other chemicals were obtained from Sigma (St. Louis, MO) and were of the finest quality available.

Cell Culture. Mycoplasma-free BS-C-1 (African Green Monkey kidney) cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 43 and were routinely cultured on polystyrene or glass coverslips in Eagle's MEM supplemented with Earle's BBS and 10% FBS. These cells were uniformly tested between passages 46 and 56 to minimize the effects of phenotypic variation in continuous culture. Primary cultures of rabbit renal proximal tubule cells (RPTC) were prepared, characterized, maintained, and tested as described previously (*27*). Cell monolayers were routinely grown to confluence at 37 °C in 5% CO_2 before testing.

Expression Plasmids. The mammalian expression plasmids pEGFP-N1 (Clontech; Palo Alto, CA) and pSV•β-gal (Promega; Madison, WI) were obtained commercially. pEGFP-N1 contains an enhanced, red-shifted F64L/S65T GFP reporter gene mutant, GFPmut1 (*24*), under the control of a human cytomegalovirus immediate early promoter and enhancer. The pSV•β-gal vector contains an SV40 viral promoter and enhancer ligated to a β-galactosidase reporter gene.

Gene Transfer. Transgene delivery into both RPTC and BS-C-1 cells was accomplished by lipofection using LipofectAMINE (GibcoBRL) according to the manufacturer's recommendations. Optimal transfection efficiency was achieved by incubating 35 mm cell monolayers with serum-free growth medium supplemented with 10 μL of LipofectAMINE and 1 μg of total plasmid DNA. Plasmid DNA was routinely purified by sequential ion exchange chromatography (Qiagen; Chatsworth, CA) and isopycnic banding in a continuous CsCl gradient before transfection. Following gene transfer, cells were maintained in 5% CO_2 at 37 °C and were serially monitored for GFPmut1 expression by fluorescence microscopy. Transient transfection efficiency was also monitored in paired control cell monolayers transfected with pSV•β-gal. These cells were fixed and assayed histochemically for β-galactosidase expression as described previously (*28*). Stably overexpressing BS-C-1 cells were selected by G418 resistance (400 μg/mL) conferred by a coexpressed neomycin resistance (*neo*^{R}) gene in pEGFP-N1. Individual stable transfectants were isolated by limiting dilution and were subsequently expanded and maintained in normal growth medium supplemented with G418 (200 μg/mL). The observations reported herein were made in at least three independent stable transfectant lineages. GFPmut1-expressing cells were morphologically indistinguishable from β-galactosidase-expressing cells or their untransfected counterparts. Stably transfected cells were uniformly tested within 10 passages of isolation.

Spectrophotometric and Spectrofluorometric Analysis. Absorption spectra of cell lysates were measured at room temperature using a Beckman DU 640 spectrophotometer (Beckman; Fullerton, CA). Spectrofluorometric analysis was performed at 37 °C using a PTI RatioMaster spectrofluorometer equipped with a 75 W xenon arc lamp, excitation and emission monochromators, a Model 710 photomultiplier tube (PMT) for photon-counting, and FeliX version 1.1 software for fluorescence analysis (Photon Technology International; South Brunswick, NJ). Fluorescence spectra were not corrected for individual xenon lamp or PMT characteristics, but all major observations were complemented or confirmed using a Perkin-Elmer Model 650-40 fluorescence spectrophotometer equipped with a 150 W xenon arc lamp, emission and excitation monochromators, and a matched PMT (Perkin-Elmer; Norwalk, CT). GFPmut1-expressing cell monolayers were analyzed for fluorescence emission at individual wavelengths between 510 and 570 nm following monochromatic excitation at or near maxima established by excitation scanning. Fluorescence data were expressed both as raw data in counts/s and as the ratio of pH-sensitive to pH-insensitive fluorescence measured following excitation at wavelengths known to be pH-sensitive (typically ∼400 nm or ∼500 nm) and pH-insensitive (typically ∼330 nm), respectively. GFPmut1-expressing cells were routinely evaluated in parallel with untransfected or β-galactosidase-expressing cells loaded with BCECF as described previously (*27*).

Fluorescence Microscopy. The PTI RatioMaster system was also used for fluorescence microscopy via a Model D-104B single channel microscope photometer attached to the optics of an Olympus Model IX50 inverted brightfield/phase contrast microscope. Fluorescence microscopy was also performed using a Carl Zeiss LSM laser scanning confocal microscope system (Carl Zeiss, Thornwood, NY). Matched short-pass cutoff and long-pass cuton filter sets designed for FITC detection were employed to minimize the contributions of both autofluorescence and spectral overlap, thereby increasing the specificity of the observed changes in fluorescence intensity.

Intracellular pH Analysis. NBC activity and pH_i were measured spectrofluorometrically using the pH-sensitive fluorophore 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) as its caged triacetoxymethyl ester (BCECF-AM) and as described previously (*27*, *29*). Confluent cell monolayers were routinely loaded with 4 μM BCECF-AM for 30 min at 37 °C before testing. This resulted in uniform loading of all cell monolayers. Changes in cellular fluorescence were calibrated by rapid equilibration of intracellular and extracellular pH in the presence of both the K^+-H^+ ionophore nigericin (4 μg/mL) and 140 mM extracellular K^+ (*30*). pH-sensitive changes in BCECF fluorescence were monitored at 530 nm following excitation at 500 nm as described (*27*, *29*). Clonal isolates of GFPmut1-expressing cells were similarly analyzed for pH-sensitive changes in fluorescence at wavelengths in the 510−570 nm range following following excitation at either 400 or 500 nm. pH-insensitive fluorescence resulting from excitation at 325−333 nm was monitored concomitantly when analyzing data by the ratiometric method. In separate experiments, 1 μM valinomycin, which is known to augment mitochondrial pH gradients (*30*), was added to the perfusate and evaluated for the ability to alter basal and stimulated whole cell fluorescence.

Subcellular Fractionation. GFPmut1-expressing cells were lysed in phosphate-buffered saline (PBS), pH 7.4, by brief sonication (∼30 s at 4 °C). After centrifugation for 1 h at 4 °C and at 100000*g*, the resulting supernatant was taken as the cytosolic fraction and was analyzed for changes in fluorescence emission over the physiologic pH range (pH 6.2−8.0). To control for influences by lysate composition, spectrofluorometric and spectrophotometric analyses were also performed following lysis in isosmotic buffered solutions containing either 100 mM KCl or 0.25 M sucrose. All lysis solutions were uniformly Ca^{2+}- and Mg^{2+}-free and were tested in both the presence and absence of detergents; 5 μg/mL aprotinin and 0−1 mM PMSF were routinely added to lysates to inhibit protease activity.

Data Analysis. Statistical comparisons were performed by analysis of variance (ANOVA) with Bonferroni Dunn correction or, where appropriate, by paired *t*-testing using StatView 4.51.2 software for Macintosh computers (Abacus Concepts) and a 95% significance level.

RESULTS

GFPmut1 Can Be Heterologously Expressed in Renal Epithelial Cells and Can Be Detected Spectrofluorometrically. Lipofection-mediated gene transfer into both BS-C-1 cells and cultured RPTC resulted in similar levels of detectable reporter gene expression. Approximately 10−20% of cells transiently transfected with pEGFP-N1 exhibited green fluorescence when transilluminated with near-visible UV light (data not shown). Similar levels of β-galactosidase reporter gene expression were observed in cells transfected with a pSV•β-gal control vector. Both mock-transfected cells and control cells expressing β-galactosidase exhibited no background fluorescence and behaved like untransfected cells when loaded with BCECF (data not shown).

Heterologous GFPmut1 Is Predominantly Cytosolic in Distribution and Is Uniformly Expressed in Clonal Isolates of Stably Overexpressing Transfectants. To enrich for GFPmut1-expressing cells and control for variations in transgene expression, individual G418-resistant colony-forming units were isolated by limiting dilution before expansion, propagation, and testing. Although the fluorescence intensity varied slightly between isolates, a given lineage exhibited uniform fluorescence intensity when excited with near-visible UV light. In addition, GFPmut1-expressing cells microscopically exhibited diffuse cytosolic and nuclear fluorescence when viewed using matched FITC filter sets. This pattern of fluorescence was indistinguishable from that observed in transient transfectants (data not shown). Over 80% of the total fluorescence of GFPmut1-expressing cell lysates was consistently recovered in 100000*g* supernatants following differential centrifugation, suggesting that GFPmut1 is predominantly cytosolic in distribution.

GFPmut1 Exhibits pH-Sensitive Fluorescence in Vitro. The spectral characteristics of soluble GFPmut1 expressed in BS-C-1 cells were consistent with those previously reported for this variant following heterologous expression in bacteria (*24*). Absorption spectra of cytosolic preparations from GFPmut1-expressing BS-C-1 cells revealed an absorption peak at 492 nm with a broad shoulder at 470 nm that was not observed in untransfected BS-C-1 control lysates (Figure 1A). Fluorescence excitation spectra of the same samples consistently revealed three closely associated excitation peaks at 472, 480, and 494 nm (Figure 1B). The absence of comparable fluorescence by either control cell lysates or homogenization buffer alone suggested that these excitation peaks were specific for GFPmut1. When excited at or near 490 nm, the soluble fraction of GFPmut1-expressing cell lysates exhibited pH-sensitive fluorescence at 510 nm over the physiologic pH range (pH 6.4−8.0; Figure 1C). These wavelengths correspond to previously reported in vitro emission and excitation maxima (488 and 507 nm, respectively) for GFPmut1 (*24*). Similar results were obtained when emission was monitored at 530 nm following excitation at 500 nm (data not shown), wavelengths employed previously to monitor pH-sensitive changes in BCECF fluorescence intensity (*27*).

GFPmut1 Also Exhibits pH-Sensitive Fluorescence in Situ Following Heterologous Expression in Cultured Mammalian Cells. To test whether these spectral characteristics were preserved in situ, we examined intact GFPmut1-expressing BS-C-1 cell monolayers for similar pH-sensitive fluorescence emission. As depicted in Figure 2A, the fluorescence excitation spectra of GFPmut1-expressing cell monolayers consistently revealed two prominent pH-sensitive regions when monitored at individual wavelengths in the 510−570 nm range [corresponding to the reported emission maximum and associated emission shoulder of GFPmut1 (*20*)]. A single excitation peak was observed at 400 nm (region I: peak *a*, Figure 2A,B) as well as a set of three closely associated peaks between 470 and 500 nm (region II: peaks *b*−*d*, Figure 2A,C). Both regions were pH-sensitive in the physiologic range, but each exhibited a unique fluorescence "bias" at pH extremes within this range. Alkaline-biased fluorescence intensity was observed following excitation at 400 nm, with progressively larger changes in fluorescence observed for a given pH increment as the pH was raised (Figure 2A,B). In contrast, excitation at wavelengths between 470 and 500 nm (region II) resulted in fluorescence with a corresponding acidic pH "bias", with larger incremental changes observed at acidic pH. The excitation peaks

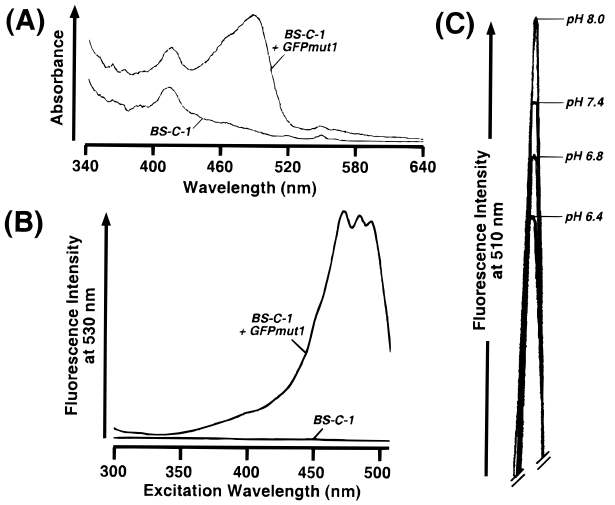

FIGURE 1: In vitro spectral characteristics and pH sensitivity of soluble GFPmut1. (A) Absorbance spectra of soluble fractions of crude lysates prepared from GFPmut1-expressing BS-C-1 cells (*BS-C-1 + GFPmut1*) and untransfected controls (*BS-C-1*). Clonal isolates of BS-C-1 cells stably expressing GFPmut1 were employed for these studies. For each sample, $\sim 10^7$ cells were collected in $\sim$3 mL of Ca^{2+}- and Mg^{2+}-free PBS, pH 7.4, supplemented with 5 μg/mL aprotinin. Following plasmalemmal disruption by brief sonication (2 × 15 s at 4 °C) in the absence of detergents, samples were centrifuged at 104000*g* and 4 °C for 1 h. Over 80% of the detectable fluorescence was consistently recovered in the resulting supernatant. Total protein contents of the depicted samples were roughly equivalent ($\sim$0.5 mg/mL). All scanning spectrophotometric analysis was performed at room temperature. (B) Fluorescence excitation spectra of the same samples depicted in (A). Excitation spectra were generated by monitoring fluorescence emission at 530 nm at 37 °C. Results from a single experiment are depicted and are representative of those observed in at least two other independent experiments. Spectra were not corrected for either illumination source or PMT characteristics, so peaks in the output spectrum of the xenon arc lamp may account for the presence of multiple peaks near the expected excitation maximum at 488 nm. (C) pH-sensitive fluorescence emission by soluble extracts of GFPmut1-expressing BS-C-1 cells. Peak outputs at 510 nm are depicted for a number of pH values within the physiologic range when excited at the excitation maximum of GFPmut1. These superimposed traces were obtained from a single experiment, which was repeated at least twice with identical results.

at 400 and 494 nm correspond to the reported excitation maxima of wild-type GFP and GFPmut1, respectively. A less prominent excitation peak at 307 nm also appeared pH-sensitive when fluorescence was monitored at 570 nm (Figure 2B), but this peak was not further evaluated.

The 400 nm excitation peak was consistently observed in scans of intact GFPmut1-expressing cells (Figure 2A,B)—but not in scans of soluble preparations from the same cells (see Figure 1B). Since this peak has not previously been reported for GFPmut1, we also tested particulate fractions of GFPmut1-expressing cell lysates for spectral changes at this wavelength. Following cell lysis under conditions known to preserve general organellar integrity, the particulate fraction was collected and resuspended in an isosmotic physiologic saline solution. Excitation scanning of these suspensions revealed excitation peaks in the 470−500 nm range indistinguishable from those observed in scans of intact GFPmut1-expressing cells and soluble preparations derived from these cells (data not shown). However, no spectral changes at or near 400 nm were observed in vitro, even at maximum sensitivity. To test the influence of lysis buffer composition on these findings, lysates were also prepared in isosmotic buffered solutions containing 100 mM KCl or 0.25 M sucrose. Excitation scanning of these preparations also failed to reveal 400 nm spectral changes at protein concentrations as high as 0.74 mg/mL (data not shown).

GFPmut1 Fluorescence Can Be Used To Probe pH_i. To extend these observations and to assess the feasibility of quantitatively estimating pH_i by monitoring GFPmut1 fluorescence in situ, we examined GFPmut1-expressing cells in parallel with paired untransfected or β-galactosidase-expressing cells loaded with the pH-sensitive chromophore BCECF. When fluorescence emission was monitored in GFPmut1-expressing BS-C-1 cells following excitation at 400 nm, rapid and reversible pH-dependent changes in fluorescence intensity were observed in the presence of extracellular nigericin and 140 mM K^+ to equilibrate pH across the plasma membrane (Figure 3, curve A). In contrast, no pH-sensitive changes in fluorescence were observed in these cells when excited with monochromatic light at wavelengths between 325 and 333 nm (Figure 3, curve B). Thus, wavelengths in this range were considered pH-insensitive in experiments evaluating pH_i by the ratiometric method. Similar results were observed in RPTC transiently transfected with pEGFP-N1 and excited at 500 nm (Figure 3, inset). These observations in both transiently and stably transfected cell monolayers reflected a range of fluorescence intensities spanning several orders of magnitude with comparable results (data not shown). Regression analysis of in situ calibration curves suggested a semilogarithmic relationship between pH_i and GFPmut1 fluorescence intensity following excitation at either 400 or 500 nm, and such analysis consistently resulted in values of $r^2 \geq 0.960$ (r = correlation coefficient). To facilitate direct comparison and to address the feasibility of monitoring pH-sensitive GFPmut1 fluorescence using parameters previously established for pH_i estimation using BCECF, we monitored both BCECF and GFPmut1 fluorescence at 530 nm following monochromatic excitation at 500 nm as described previously (*27*). When examined under conditions of changing pH_i known to primarily reflect NBC activity, both BS-C-1 cells and RPTC expressing GFPmut1 exhibited changes in whole cell fluorescence that were indistinguishable from those observed in untransfected cells loaded with BCECF (Figure 4). Mitochondrial alkalinization by valinomycin also failed to increase cellular fluorescence (data not shown), suggesting that mitochondrial pH transitions are not reflected by changes in GFPmut1 fluorescence. Parallel fluorometric assays using both GFPmut1 and BCECF resulted in identical estimates of basal and CO_2-stimulated Na/HCO_3 cotransport activities for each cell type (Figure 5). Identical pH_i measurements obtained using each fluorophore thus validated the utility of heterologous GFPmut1 as an indicator of intracellular, largely cytoplasmic, pH.

DISCUSSION

We have demonstrated that the fluorescence of the red-shifted F64L/S65T mutant, GFPmut1, is pH sensitive, both in vitro and in intact cells. Comparable observations both in vitro and in situ suggest that in situ changes in fluorescence reflect environmental interactions (e.g., protonation−deprotonation events, conformational changes, or oligomerization) rather than alterations in GFPmut1 transgene expression. Environmental influences such as temperature, ionic strength,

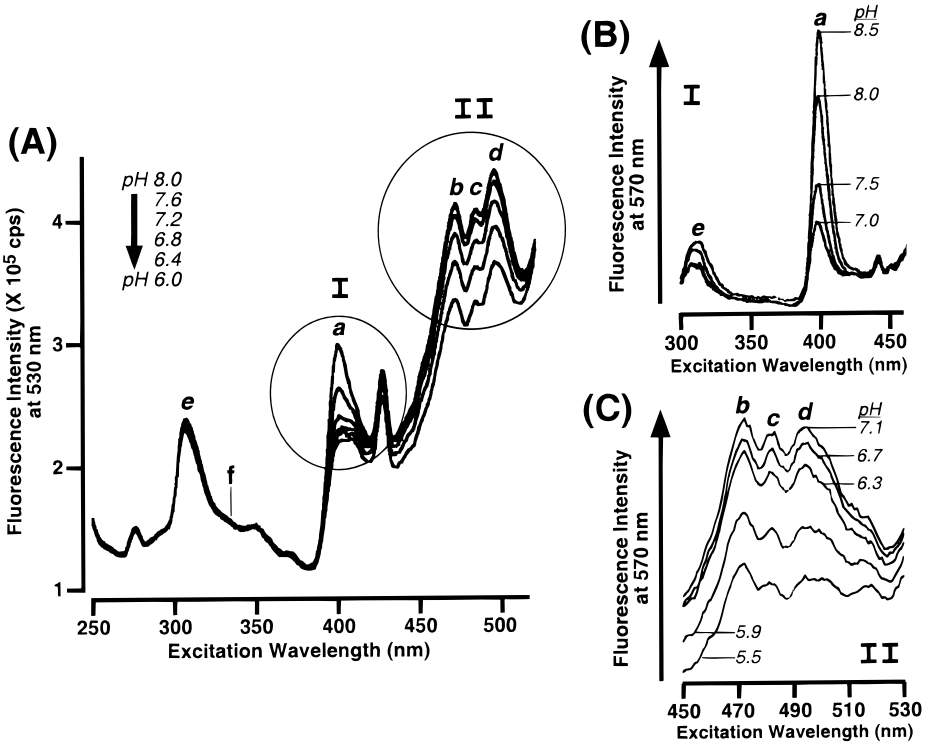

FIGURE 2: Fluorescence excitation spectra of GFPmut1 in situ. (A) Uncorrected fluorescence excitation spectra of GFPmut1-expressing BS-C-1 cells consistently revealed two pH-sensitive regions (I and II, circled) when examined over the physiologic range. Region I contained a peak (peak *a*) corresponding to the "wild-type" 400 nm excitation peak and resulted in alkaline-biased fluorescence. Region II contained three closely associated excitation peaks between 472 and 494 nm (peaks *b*, *c*, and *d*) corresponding to those observed in vitro (Figure 1B). The lowest frequency peak at 494 nm approximates the reported excitation maximum of GFPmut1 in bacterial lysates (*24*). Closely associated peaks at 472 and 480 nm likely reflect peaks in the xenon arc lamp output spectrum (*29*), but the presence of higher energy excited states cannot presently be excluded. Previous reports of infrequent spectral changes in vitro at or near these wavelengths have been attributed to metastable intermediates and are consistent with the latter possibility. To emphasize the unique pH bias of each region, cells were examined under conditions found to exaggerate pH-sensitive changes in each region: the changes characteristic of regions I and II are depicted in (B) and (C), respectively. Some excitation scans suggested that peak *e* may also exhibit pH sensitivity, although this was not examined further. The pH-insensitive excitation wavelength used in the present studies is also marked (see *f*).

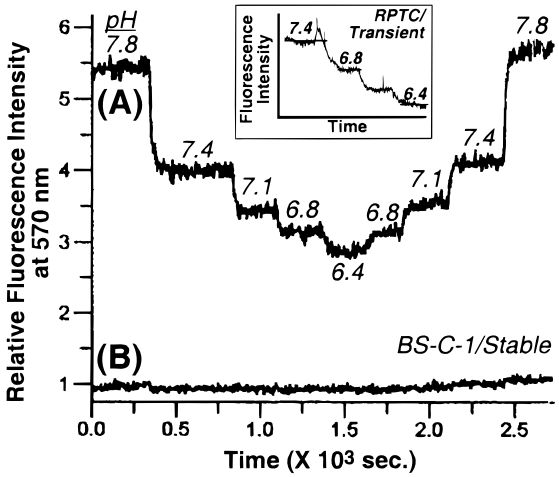

FIGURE 3: pH calibration of fluorescence in renal epithelial cells expressing GFPmut1. A representative calibration curve is depicted and is typical of those observed in at least three separate experiments involving BS-C-1 cells stably expressing GFPmut1. The reversibility of these pH-dependent changes is readily apparent from the curve and represents a consistent observation in the experiments reported herein. Dual excitation wavelength monitoring at both pH-sensitive [(A) 400 nm] and pH-insensitive [(B) 330 nm] excitation wavelengths is depicted. Similar results were obtained with RPTC following transient GFPmut1 gene transfer (inset).

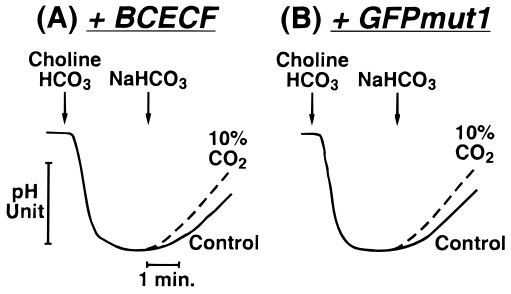

FIGURE 4: Comparison of pH-sensitive BCECF (A) and GFPmut1 (B) fluorescence in situ. NBC-dependent changes in pH_i were evaluated in BS-C-1 cell monolayers grown on coverslips and expressing GFPmut1 or loaded with BCECF. Cells were preequilibrated and perfused continuously with a chloride-free physiologic saline solution containing 25 mM $NaHCO_3$ and 1 mM amiloride. Extracellular pH was maintained constant at 7.4 throughout. Experiments were initiated by the equimolar substitution of choline·HCO_3 for $NaHCO_3$ (first arrow), which resulted in a rapid decline in pH_i attributable to decreased NBC activity (*27*). Following replacement of $NaHCO_3$, the initial rate of pH_i recovery was taken as the rate of NBC activity and was monitored in both control cells (solid line) and following the adaptive increase in NBC activity resulting from acute stimulation with 10% CO_2 (dashed line) (*27*). During $NaHCO_3$ readdition (second arrow), the chart speed was accelerated to facilitate accurate measurement of changes in pH_i. The fluorescence data depicted were normalized to facilitate comparison of GFPmut1 with BCECF.

and protein concentration were also rigorously controlled for in vitro and suggest that changes in ambient pH were sufficient to explain our observations. To properly interpret pH-sensitive GFPmut1 fluorescence in situ, we microscopically examined both RPTC and BS-C-1 cells expressing this

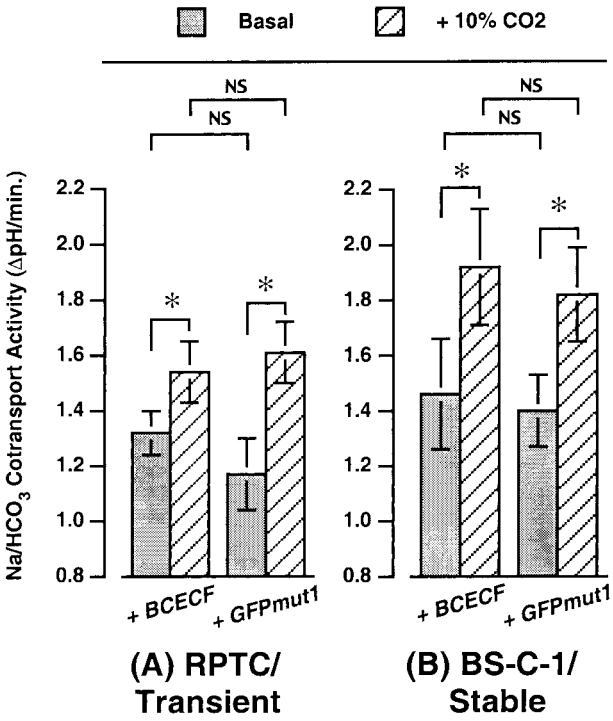

FIGURE 5: Comparison of fluorometric assays of basal and stimulated NBC activity using BCECF and GFPmut1 in RPTC and BS-C-1 cells. (A) RPTC transiently expressing GFPmut1 were compared to paired untransfected cells loaded with BCECF. Data from seven separate experiments are depicted as the mean ± SEM. (B) BS-C-1 cells stably expressing GFPmut1 were similarly compared with paired untransfected cell monolayers loaded with BCECF. Data from eight separate experiments are depicted as the mean ± SEM. Statistical significance was assessed by paired *t*-testing at a 95% confidence level.

protein and observed diffuse cytosolic and nuclear fluorescence, consistent with the reported intracellular distribution of heterologously expressed wild-type GFP (*4, 6, 11, 12*). Fluorescence microscopy also suggested that the majority of GFPmut1 resides in the cytosol. Consistent with this interpretation, over 80% of the total cell fluorescence was recoverable in soluble fractions of GFPmut1-expressing cell lysates. Taking both nuclear size and fluorescence into account, it is reasonable to speculate that nuclear GFPmut1 accounts for the vast majority of noncytosolic fluorescence in these cells. The nuclear envelope would not normally be expected to maintain a significant pH gradient in quiescent, confluent cell monolayers (*29*). Since other intracellular organelles capable of maintaining a substantial pH gradient constitute, at most, only a few percent of the total cell volume, they should minimally influence total cellular fluorescence (*29*). However, to test whether mitochondrial pH transitions contributed to our observations, we treated cells with the potassium ionophore valinomycin, which is known to increase mitochondrial pH and thereby affect the fluorescence of pH-sensitive fluorophores that distribute into the mitochondrial matrix (*30*). The failure of valinomycin to increase pH-sensitive fluorescence in situ suggested that the observed changes primarily reflect cytosolic, and not mitochondrial, pH transitions. The uniform agreement between pH_i estimates using both GFPmut1 and the cytosolic fluorophore BCECF further validates these conclusions.

pH_i estimation using GFPmut1 provides a number of important technical advantages over previous methods employing chemical fluorophores. Although only a small fraction (10−20%) of cells exhibited demonstrable fluorescence following transient transfection with pEGFP-N1, there was uniform agreement between measured changes in pH_i in these cells and in their stably transfected counterparts. Since there was no background fluorescence, estimates of pH_i reflected only that subset of cells expressing the GFPmut1 transgene. In the homogeneous cultured cell models employed for our experiments, this subset could be taken as representative of the whole. Transient GFPmut1 expression was both prolonged and robust, with maximum fluorescence occurring within 24−36 h of gene transfer and persisting for at least 3−5 days. The sensitivity of pH_i monitoring with transiently expressed GFPmut1 was uniformly comparable or superior to that of BCECF in these cells and was markedly superior in stably transfected cells. Thus, serial pH_i measures are feasible not only in stable transfectants but also in cells that transiently express GFPmut1, providing an opportunity to study chronic pH_i changes in primary cell cultures where stable gene transfer is not possible. GFPmut1 is completely retained within intact cells, and variations in expression can be minimized by clonal selection of stable transfectants. Thus, uniform fluorophore loading is also achievable and obviates the loading and leakage problems encountered with pH-sensitive chemical chromophores such as BCECF. This makes the present method less reliant on ratiometric comparisons at pH-sensitive and pH-insensitive wavelengths. Identical results obtained using both absolute photon counts and ratiometric methods are compatible with this interpretation. In addition, the immediately reversible nature of these pH_i-sensitive changes and the quantitative recovery of fluorescence for a given pH level do not suggest that underlying quenching or photobleaching are significant contributors to our observations. Consistent with this interpretation, we observed very little GFPmut1 photobleaching following prolonged excitation (≤1 h) at 488 nm during microscopic examination (R. B. Robey, unpublished observations). Although specialized filter sets are commercially available for a number of GFPs, the ability to adequately detect and quantitate GFPmut1 fluorescence using the same spectrofluorometric parameters and equipment employed to monitor BCECF also obviates the need for a specialized detection system (*24*).

Both wild-type GFP and GFPmut1 exhibit fluorescence emission maxima at 507−508 nm in vitro, and our observations in situ were in agreement with these findings. Fluorescence excitation spectra of soluble GFPmut1 were also consistent with those described previously for this variant (*24*). The fluorescence excitation spectrum of heterologously expressed GFPmut1 in situ, however, differed significantly from that observed in vitro in one important regard. GFPmut1 uniformly exhibited a pH-sensitive excitation peak at 400 nm in intact cells that was not observed following cell lysis. This peak, which has not previously been reported for GFPmut1, corresponds to the wild-type peak associated with the neutral protonated form of the native chromophore (*22, 23, 31*) and exhibits similar alkaline enhancement of fluorescence (*17*). Deprotonated anionic chromophore development has been proposed to account for the secondary ∼470 nm excitation peak of the wild-type GFP, as well as the corresponding principal excitation peaks of a number of red-shifted GFP variants such a GFPmut1. Since structural

and functional analysis of GFPs has suggested a consistent relationship between chromophore conformation and spectral features (*23*), our findings may have important biophysical implications (vide infra).

In attempting to correlate our observations in situ with previous observations in vitro, it is important to note that S65 substitutions uniformly result in loss of the 400 nm "wild-type" excitation peak, either alone (*32*) or in combination with other mutations (*23*, *24*). An isolated S65T mutation also results in accelerated chromophore maturation (*32*) and a 6-fold increase in fluorescence intensity (*33*). In combination with an F64L substitution, fluorescence intensity is increased as much as 35-fold (*24*). Although an isolated F64L substitution has no effect on either the spectral characteristics or the crystal structure of GFP, both mutations occur within the defined hexapeptide chromophore (*34*), and both are necessary for maximal fluorescence of GFPmut1 (*24*). The contributions of the F64L substitution to the enhanced fluorescence of GFPmut1 are presumed to occur via mechanisms such as more efficient protein folding or increased solubility (*23*, *24*). The uniform detection of a prominent 400 nm excitation peak in GFPmut1-expressing intact cells, but not in lysates prepared from these cells or in controls, is therefore of great interest and suggests that this peak is specific for GFPmut1 in situ. Identical spectral characteristics of lysates prepared in physiologic saline, buffered isosmotic KCl, and isotonic buffered sucrose suggest that the loss of this peak in vitro is not attributable to lysis buffer composition. These findings also exclude intracellular K^+ concentration as a trivial explanation for our observations in situ. The recovery of wild-type spectral features in situ that are lost in vitro following mutagenesis suggests additional determinants of GFP fluorescence in vivo. However, without specific knowledge of both the crystal structure(s) of GFPmut1 and the spectral characteristics of other S65-substituted variants in situ, it is not clear whether these additional determinants are generally applicable to all GFPs or are specific for certain variants. The recently reported in vitro spectral characteristics of another "strong green" S65-substituted variant, sg25 [F64L/S65C/I167T/K238N (*23*)], may provide important clues to the mechanisms underlying these observations. Like GFPmut1, sg25 does not typically exhibit a "wild-type" excitation peak at 400 nm, but an excitation shoulder develops at this wavelength as protein content is raised in vitro. Since elevated protein concentrations favor GFP dimerization, which has been postulated to play an important role in GFP function in vivo (*23*), this may suggest a mechanism whereby in situ spectral characteristics may differ from those observed in vitro. A common F64L substitution in both GFPmut1 and sg25 also makes it attractive to speculate that this substitution may play a permissive role in the formation or recovery of a "wild-type" conformation that is otherwise disrupted by S65 substitutions. Consistent with this hypothesis, crystallographic analysis of sg25 has revealed the presence of alternate chromophore conformations, one of which is similar to that observed for wild-type GFP (*32*). Taken together, these findings suggest that the fluorescence characteristics of GFPmut1 previously attributed to anionic chromophore formation may have additional biophysical determinants in vivo.

In summary, we have successfully exploited the in situ pH sensitivity of GFPmut1 to estimate pH_i in cells that heterologously express this variant, and we have validated this fluorometric assay by direct comparison with a conventional assay employing BCECF. In addition, we have demonstrated that GFPmut1 exhibits novel spectral features in situ, findings with important implications for both pH_i estimation and the understanding of GFP fluorescence in vivo. Although the underlying molecular mechanisms are not well understood, these findings provide the basis for a novel and important experimental application for this class of bioluminescent proteins, and for GFPmut1 in particular. In practical terms, they also provide a simple and economical alternative to the use of chemical fluorophores (e.g., BCECF) for pH_i monitoring in real time. These findings further raise the possibility of using GFPs as dual function reporter genes when coexpressed with or fused to other transgenes, permitting simultaneous real-time evaluation of transgene expression and related effects on intracellular pH. Nondisruptive pH-sensitive GFP chimeras would be particularly useful in this regard, since their expression could provide direct insight into the relationships between the intracellular distribution and expression level of heterologous transgenic peptides and related changes in pH.

ADDED IN PROOF

Since the original submission of this paper, we became aware of an independent report of the use of GFPmut1 as a probe of pH_i (*35*). In this report, the pH sensitivities of a number of GFP variants, including GFPmut1, were characterized in vitro, and GFPmut1 fluorescence was monitored as an indicator of local pH following targeting to specific intracellular compartments. Although no comparisons were made with other methods of pH_i estimation and in situ spectral characteristics were otherwise not addressed, their observations are in agreement with our findings and validate the utility of GFPmut1 as an indicator of pH_i.

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