Cellular Characterization of Adenylate Kinase and Its Isoform: Two-Photon Excitation Fluorescence Imaging and Fluorescence Correlation Spectroscopy

Qiaoqiao Ruan,*† Yan Chen,* Enrico Gratton,* Michael Glaser,† and William W. Mantulin*†
*Laboratory for Fluorescence Dynamics, Department of †Biochemistry, University of Illinois in Urbana-Champaign, Urbana, Illinois 61801 USA

ABSTRACT Adenylate kinase (AK) is a ubiquitous enzyme that regulates the homeostasis of adenine nucleotides in the cell. AK1β (long form) from murine cells shares the same protein sequence as AK1 (short form) except for the addition of 18 amino acid residues at its N-terminus. It is hypothesized that these residues serve as a signal for protein lipid modification and targeting of the protein to the plasma membrane. To better understand the cellular function of these AK isoforms, we have used several modern fluorescence techniques to characterize these two isoforms of AK enzyme. We fused cytosolic adenylate kinase (AK1) and its isoform (AK1β) with enhanced green fluorescence protein (EGFP) and expressed the chimera proteins in HeLa cells. Using two-photon excitation scanning fluorescence imaging, we were able to directly visualize the localization of AK1-EGFP and AK1β-EGFP in live cells. AK1β-EGFP mainly localized on the plasma membrane, whereas AK1-EGFP distributed throughout the cell except for trace amounts in the nuclear membrane and some vesicles. We performed fluorescence correlation spectroscopy measurements and photon-counting histogram analysis in specific domains of live cells. For AK1-EGFP, we observed only one diffusion component in the cytoplasm. For AK1β-EGFP, we observed two distinct diffusion components on the plasma membrane. One corresponded to the free diffusing protein, whereas the other represented the membrane-bound AK1β-EGFP. The diffusion rate of AK1-EGFP was slowed by a factor of 1.8 with respect to that of EGFP, which was 50% more than what we would expect for a free diffusing AK1-EGFP. To rule out the possibility of oligomer formation, we performed photon-counting histogram analysis to direct analyze the brightness difference between AK1-EGFP and EGFP. From our analysis, we concluded that cytoplasmic AK1-EGFP is monomeric. fluorescence correlation spectroscopy proved to be a powerful technique for quantitatively studying the mobility of the target protein in live cells. This technology offers advantages in studying protein interactions and function in the cell.

INTRODUCTION

Adenylate kinase (AK) is a ubiquitous monomeric enzyme that catalyzes the following reaction: Mg^{2+}-ATP + AMP $\leftrightarrow$ Mg^{2+}-ADP + ADP (Noda, 1973). The cell uses this reaction to convert AMP to ADP (Glaser et al., 1975), thereby regulating adenine nucleotide levels. AK is also involved in other reactions, such as the biosynthesis of phospholipids (Goelz and Cronan, 1982). In vertebrates, three isozymes of AK have been characterized: AK1 is cytoplasmic, AK2 is localized in the intermembrane space of mitochondria, and AK3 is localized in the mitochondrial matrix. Recently, a long form of AK1 protein from murine cells, AK1β, was identified. Its amino acid sequence was identical to that of the murine cytoplasmic AK1, except for the addition of 18 amino acids (MGCCVSSEPQEE-GGRKTG) at the N-terminus. The transcription level of the AK1β gene was found to be upregulated by p53, whereas that of the AK1 gene was not. This observation suggested that AK1β might have some novel biological functions other than its conventional function of regulating nucleotide levels. For example, it may be involved in the cell cycle arrest process (Collavin et al., 1999). We are interested in the effect of the additional 18 amino acid residues on the biological function of AK1β. It was hypothesized that the 18 additional residues might serve as a signal for protein lipid modification and targeting of the protein to the plasma membrane, based on the N-myristoylation consensus motif. Protein N-myristoylation is the result of the co-translational addition of myristic acid to a Gly residue at the extreme N-terminus after removal of the initiating Met (Utsumi et al., 2001). In general, to direct protein N-myristoylation, the N-terminal consensus motifs such as Met-Gly-X-X-X Ser-X-X-X (Johnson et al., 1994) or Met-Gly-X-X-X Thr-X-X-X (Boutin, 1997) are preferred.

Fluorescence correlation spectroscopy (FCS) was first introduced by Webb and his coworkers in 1972 (Magde et al., 1972). It has evolved as a powerful method to study particle dynamics on a single-molecule level in part because of recent technological advances (such as confocal microscopy, multiphoton laser excitation). FCS uses the autocorrelation function to characterize fluorescence intensity fluctuations in the observation volume and it can be used to study particle diffusion (Fahey et al., 1977; Koppel et al., 1976), chemical kinetics (Haupts et al., 1998; Starr and Thompson, 2001), and molecular aggregation (Palmer and Thompson, 1987; Qian and Elson, 1990). In recent years, several laboratories have introduced FCS studies on live cells. Fluorescently labeled particles were first loaded into cells or labeled on the surface of the cells; the dynamics of the fluorescent dyes in the cells were then studied with FCS. Successful application of FCS in the cell provides a new experimental approach to quantitatively study the diffusion rates of cellular mole-

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Address reprint requests to Dr. William W. Mantulin, 1110 W. Green St., Urbana, IL 61801-3080. Tel.: 217-244-5620; Fax: 217-244-7187; E-mail: mantulin@uiuc.edu.

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cules or components (Berland et al., 1995; Schwille et al., 1999a; Brock et al., 1998; Politz et al., 1998). With the discovery of green fluorescence protein (GFP) and the development of molecular engineering, a protein of interest can be easily fused to GFP and expressed in the cell. The dynamics of the target protein can then be followed with FCS (Wachsmuth et al., 2000; Brock et al., 1999; Hink et al., 2000). GFP is a 27-kDa monomer first found in the jellyfish *Aequorea* (Shimomura et al., 1962). The gene for GFP has been isolated. It has become a useful tool for making chimeric proteins, where GFP acting as a fluorescent reporter is linked to proteins of interest. GFP tolerates N- and C-terminal fusion to a broad variety of proteins. The fusion proteins, which are fluorescent, usually maintain normal function and cellular localization of the host protein (Tsien, 1998). Enhanced GFP (EGFP) is a GFP mutant with improved optical properties and folding stability. Careful investigation of the spectral properties of EGFP (brightness, resistance to bleaching, and long wavelength emission) suggests it is a superior molecular indicator for quantitative fluorescence microscopy studies of intra- and intercellularly tagged-proteins (Cinelli et al., 2000).

In our system, we constructed AK1-EGFP and AK1β-EGFP chimera proteins and then expressed them in HeLa cells. Two-photon excitation scanning fluorescence images of the transfected cells directly revealed the localization of the chimera proteins in the cells. AK1β-EGFP mainly localized on the plasma membrane, whereas AK1-EGFP was distributed throughout the cell except for the nuclear membrane and some vesicles. We demonstrated the power of fluorescence imaging in differentiating two structurally and functionally similar proteins in the cells. The mobility of AK1 and AK1β in live cells was quantified by FCS. We discussed in detail how to carry out FCS measurements in live cells and our findings about the biological properties of the two intracellular isoforms of AK. FCS can provide important information about the microenvironment of the target protein and reveal any possible protein-protein or other cellular interactions. In addition to the FCS measurements, we also performed photon-counting histogram (PCH) analysis on the FCS data, which provided molecular brightness information and allowed for direct resolution of molecular heterogeneity (e.g., monomer and dimer mixtures). To characterize the effect of the fusion protein on the optical properties of EGFP, we also measured the fluorescence lifetime of AK1-EGFP and AK1β-EGFP in transfected cells and compared then with EGFP-transfected cells.

MATERIALS AND METHODS

Cell culture

HeLa cells obtained from American Type Culture Collection (Rockville, MD) were grown in a 5% CO_2 humidified atmosphere at 37°C in Dulbecco's modified Eagle's medium (Gibco, Gaithersburg, MD) 4.5 g/L glucose, 2 mM L-glutamine, without phenol red, 25 mM Hepes (pH 7.4),10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin (Sigma Chemical Co., St. Louis, MO).

Generation of EGFP fusion proteins and transient transfected cells

The AK1 gene fragment obtained from Dr. Schneider's group (Universidad Simon Bolivar) was inserted into the pEGFP-N1 plasmid (Clontech, Palo Alto, CA) between the *Eco*R1 and *Bam*H1 restriction sites. The stop codon for the AK1 gene (TAA) was mutated to GAA using the Quick-Change Strategene mutagenesis kit (Stratagene, La Jolla, CA). There was a seven-amino-acid-residue linker (LDPPVAT) between AK1 and EGFP. The final plasmid construct (AK1-pEGFP-N1) was sequenced to ensure that AK1 was in frame with EGFP and there was no random mutation in the chimera protein. For transient transfection, HeLa cells were seeded in an eight-chambered cover glass system (Nagle Nunc International, Rochester, NY) at a confluency of 20–30%. The next day, HeLa cells were transfected with the AK1-pEGFP-N1 plasmid DNA using the Effectene transfection kit (Qiagen, Chatsworth, CA). At 24 h after transfection, the cells were used for fluorescence microscopy measurements. For FCS measurements, the AK1-pEGFP-N1 plasmid was linearized with the *Afl*II restriction enzyme to reduce the expression level of AK1-EGFP. An activity assay for AK (Huss and Glaser, 1983) was performed on the AK1-EGFP-transfected HeLa cell lysate. It confirmed that the chimera protein was enzymatically active (data not shown).

The AK1β gene fragment was cloned into the pEGFP-N1 plasmid and transfected into HeLa cells in the same manner. As for FCS measurements, it was not necessary to linearize the plasmid. The low expression level of this chimeric protein in the cell was appropriate for FCS measurements.

Two-photon excitation fluorescence microscopy instrumentation

The two-photon excitation scanning fluorescence microscope used in the experiments was assembled in our laboratory. The optical microscope was an Axiovert 100 inverted microscope (Zeiss, Oberkochen, Germany). The objective was a Zeiss Plan Neofluar 63× (1.25 N.A., oil). A mode-locked titanium-sapphire laser with 80-MHz, 100-fs pulse width (Tsunami, Spectra-Physics, Mountain View, CA) was used as the excitation light source. The laser was guided by an *x-y* galvano-scanner (model 6350; Cambridge Technology, Cambridge, MA) to achieve beam scanning in both *x* and *y* directions. A photomultiplier tube (Hamamatsu HC120–08, Somerville, NJ) was used for light detection in the photon-counting mode. A BG39 optical filter was placed before the photomultiplier for efficient suppression of IR excitation light. Data were processed and analyzed with software developed in our laboratory (SimFCS and Globals, Window Edition). At any designated area in the cell, fluorescence intensity imaging and FCS measurements can be acquired with the above-described instrumentation.

Two-photon excitation scanning fluorescence imaging

The excitation wavelength used in our study was 915 nm, where the two-photon action cross section is maximized for EGFP and the cellular autofluorescence is relatively low (Xu et al., 1996). The fluorescence images of transfected cells were scanned at the rate of 50 μs/pixel. Each frame had 256 × 256 pixels, and each image was integrated for 10 frames.

FCS and PCH analysis

FCS is based on the principle that, when fluorescent molecules are in equilibrium, there are always spontaneous microscopic fluctuations of this equi-

librium in a small observation volume (sub-femtoliter). The fluorescence fluctuations can originate from the diffusion of fluorescent molecules through the observation volume or changes of the fluorescence quantum yield of the fluorophore due to chemical reaction. Depending on the origin of the fluctuation, the fluctuation contains information about the diffusion rate of the molecule or the rate of chemical reaction (for an overview, theory, and applications of FCS, see Rigler and Elson, 2001). In a system where particles undergo only translational diffusion, the analysis of these fluctuations allows the determination of their diffusion coefficient (D) and the average number of particles (N) in the excitation volume (Palmer and Thompson, 1987). Any changes in the diffusion coefficient of a particle in solution would reflect changes of its size (or shape) or the viscosity of the solution. Any changes in the number of particles in the excitation volume would suggest particle association or dissociation. Because the relative amplitude of the fluctuations is inversely proportional to the number of molecules simultaneously observed in the excitation volume, FCS measurements are usually carried out at nanomolar sample concentrations, where the fluctuations are observable above the average fluorescence signal.

From these spontaneous microscopic fluctuation measured directly by FCS experiments, an autocorrelation curve can be calculated using the autocorrelation function $G(\tau)$. This function reflects the time-dependent decay of the fluorescence intensity fluctuation. The normalized autocorrelation function $G(\tau)$ is defined as:

$$G(\tau) = \frac{\langle \delta F(t) \times \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2},$$

where $\delta F(t) = F(t) - \langle F \rangle$ expresses the fluctuation in fluorescence intensity at time t.

Experimental autocorrelation functions $G(\tau)$ were fitted to theoretical functions using a Gaussian-Lorentzian beam profile (Berland et al., 1995) to recover the diffusion coefficient and the number of molecules in the excitation volume.

FCS measurements in our experiments were carried out on many compartments of the cell (nucleus, cytoplasm, plasma membrane, etc.) and in many different cells. The sampling frequency was 20 kHz, and each measurement lasted less than 3 min. The laser power was 1 mW at the sample. No photobleaching was observed for cytosolic measurements, and only a small amount was observed for the membrane. The waist (ω_0) of the excitation beam was calibrated with EGFP in solution ($D = 87\ \mu m^2/s$) (Swaminathan et al., 1997) before each day's measurements. The typical values of ω_0 are at the range of 0.3–0.35 μm.

Although the temporal behavior of fluctuation can be analyzed by the autocorrelation function, the amplitude of the fluctuation is characterized by its probability distribution (PCH). PCH is experimentally determined by the histogram of the detected photons per sampling time for a fluorescence fluctuation experiment. The theoretical basis of PCH was described by Chen et al. (1999). Two parameters, the molecular brightness ϵ (in counts per second per molecule, cpsm) and the average number of molecules per observation volume (N) can be extracted from the PCH analysis based on a theoretical distribution function $\Pi(k; \bar{N}, \epsilon)$. PCH analysis is advantageous for detecting the presence of molecule dimerization or aggregation based on the brightness of the molecule when the changes in diffusion rate were too small to be conclusive. For example, the brightness of a dimer will be twice as bright as a monomer, whereas the diffusion rate of a dimer will be only 25% slower than that of a monomer. PCH analysis can resolve multiple components, if their brightness contrast is sufficient (Muller et al., 2000).

Two-photon excitation fluorescence lifetime imaging

The fluorescence lifetime measurements were performed on a frequency domain instrument assembled in our laboratory, which is integral to the two-photon excitation microscope described above. Our instrument operates at a high cross-correlation frequency that rapidly provides lifetime information on a per pixel basis. A gain-modulated photo multiplier tube (PMT) detects the fluorescence signal from each position. A second gain-modulated PMT monitors the laser beam to correct for frequency and modulation drift of the laser pulse train. The analog outputs of the two PMTs are digitized by the data acquisition computer. The excitation light was modulated at 80 MHz, and the cross-correlation frequency was set at 2500 Hz. Data were acquired at eight points per cross-correlation period. The scanner was running at the rate of 400 μs/pixel. The lifetime reference was fluorescein in 0.1 M NaOH (4.05 ns).

RESULTS

Two-photon excitation scanning fluorescence imaging of transfected cells

The fluorescence of the tagged EGFP was directly visualized in live HeLa cells with two-photon excitation scanning fluorescence microscope. Fig. 1 *A* shows fluorescence images of several representative AK1-EGFP-transfected cells. The AK1-EGFP protein was widely distributed throughout the cell, except for the nuclear membrane and some vesicular structures. The expression levels of AK1-EGFP vary from one cell to another under the same transfection conditions. For example, there was at least a twofold fluorescence intensity difference between the two adjacent cells in the first image of Fig. 1 *A*. The fluorescence intensity of the next image in Fig. 1 *A* was so high that it cannot be displayed on the same fluorescence scale as the first image. Fig. 1 *B* shows fluorescence images of several typical AK1β-EGFP-transfected cells. This fusion protein was mainly localized on the plasma membrane. In some cells, fluorescence from AK1β-EGFP was not homogeneously distributed on the plasma membrane. AK1β-EGFP was also found in the cytoplasm and the nucleus of some cells, when the overall protein expression level was high. Even though AK1β-EGFP can be expressed in HeLa cells and the protein expression levels varied significantly from cell to cell, the average expression level of AK1β-EGFP was much lower than that of AK1-EGFP. As a control experiment, the EGFP plasmid was transfected into HeLa cells. Its corresponding fluorescence image is shown in Fig. 1 *C*.

FCS

FCS measurements were carried out on healthy transfected cells with low protein expression levels. The cells shown in Fig. 1 (except *panel B*) can only be used for fluorescence imaging, because the average fluorescence is too high to resolve fluctuation at a single-molecule level. For the FCS experiments, the protein expression level must be relatively low, which can be achieved by linearizing the plasmid and optimizing the plasmid concentration. However, within the same batch of transfection, the fluorescence intensity of transfected cells still ranged from 5000 cps to millions of cps. Only cells with fluorescence intensity below 40,000 cps were selected for FCS measurements. The fluorescence

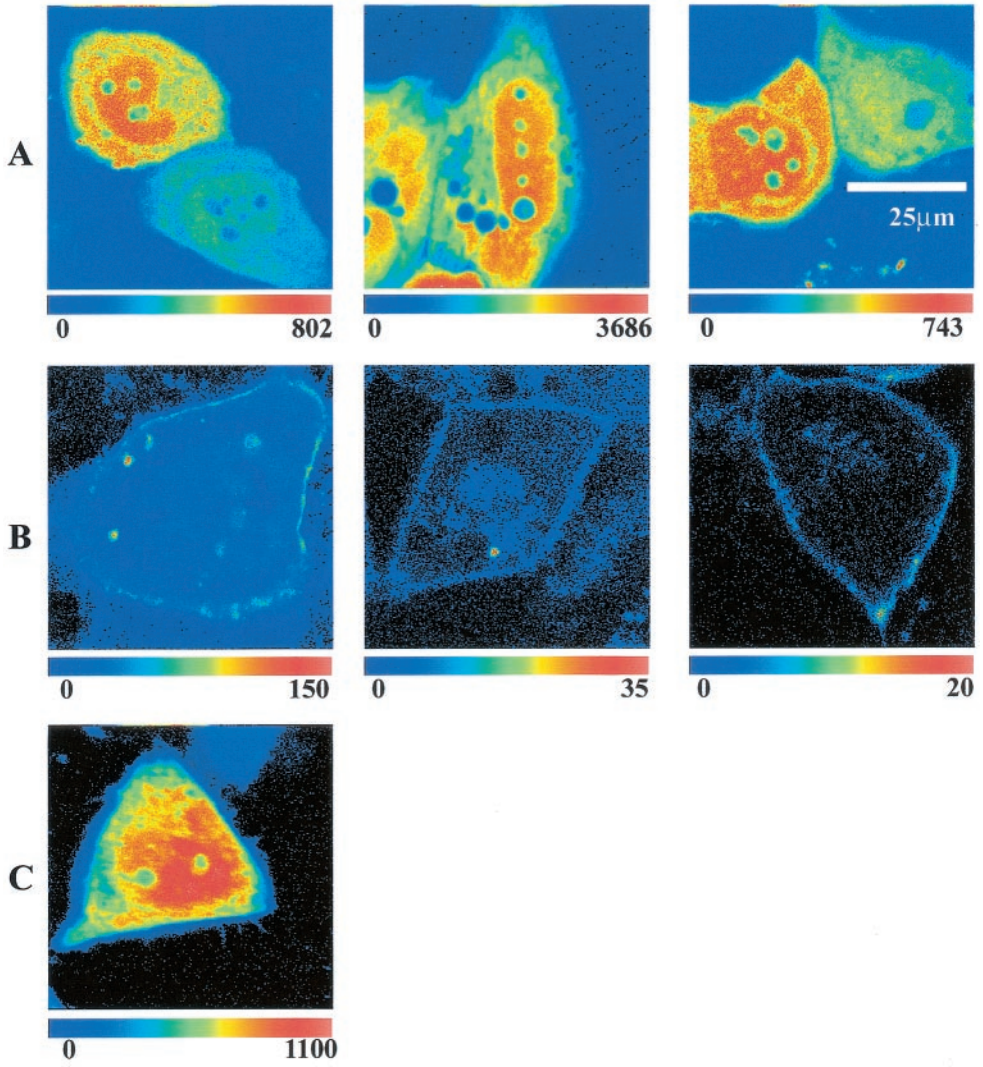

FIGURE 1 Fluorescence images of EGFP and the fusion proteins in transfected HeLa cells in pseudocolor. (*A*) AK1-EGFP; (*B*): AK1β-EGFP; (*C*) EGFP.

intensity of nontransfected cells ranged from 300 to 1000 cps. Therefore, the autofluorescence of the cell was usually less than 10% of the total fluorescence intensity measured in the transfected cell. Fig. 2 shows some examples of typical autocorrelation curves (displayed as ●, ▲, ▼, *, and ×) calculated from our FCS measurements. $G(\tau)$ was normalized to unity for ease of comparison. The lines running through the data points ●, ▲, ▼, *, and × represent the fitted autocorrelation curves. The calculated autocorrelation curves (displayed as ▲ and ●) for AK1-EGFP and AK1β-EGFP were almost identical when measured inside the cell (cytosol). Both curves could be fit with a single-component model. The fitted curves are superimposable. The recovered diffusion coefficient (D) of cytosolic AK1-EGFP and AK1β-EGFP was 13 $\mu m^2/s$. The average residence time ($\tau = \omega_0^2/8D$, for two-photon excitation) of the chimeric proteins inside the excitation volume was ~0.9 ms. When the excitation laser beam was focused on the plasma membrane of the cell, the autocorrelation curve of AK1-EGFP was similar to those measured in the cytoplasm. By contrast, the autocorrelation curve of AK1β-EGFP (×) measured in the membrane could be fitted only with a two-component model. The inset of Fig. 2 shows the two resolved components. The diffusion coefficient of the fast component was similar to that of AK1β-EGFP in the cytoplasm, whereas the second component was ~60 times smaller (D = 0.23 $\mu m^2/s$). The ratio of these two components depends on the position of the focus in the Z direction. As a control experiment, the diffusion rates of EGFP in live cells were also measured. The ▼ in Fig. 2 represents the autocorrelation curve of EGFP expressed in HeLa cells. The diffusion coefficient of EGFP in the cell was approximately three times smaller than that of EGFP (*) in solution (D = 87 $\mu m^2/s$). The average residence time of cytosolic EGFP in the excitation volume was 0.5 ms.

Live cells are complex organisms. The cellular structure, fluidity, and protein function may vary from region to region, cycle to cycle, and certainly from cell to cell. It is

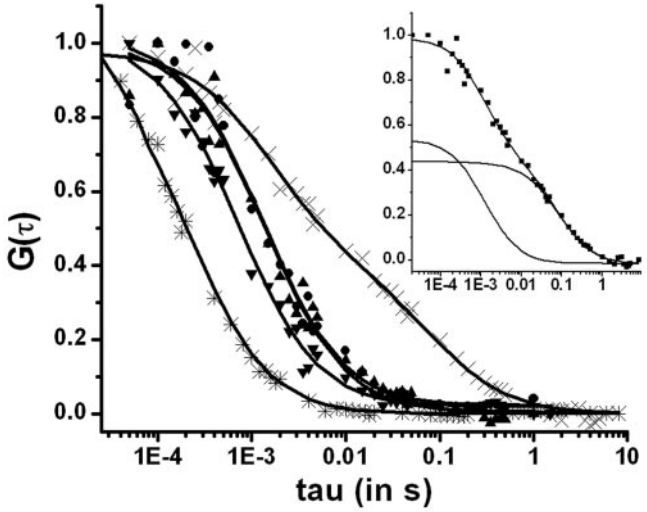

FIGURE 2 Normalized autocorrelation curves of EGFP and EGFP fusion proteins in the cell. *, EGFP in solution; ▼, EGFP in HeLa cell; ▲, AK1-EGFP in HeLa cell; ●, AK1β-EGFP inside HeLa cell; ×, AK1β-EGFP on the plasma membrane of the HeLa cell. The curves for AK1-EGFP and AK1β-EGFP are superimposed and not individually discernable. The inset shows the autocorrelation curve of AK1β-EGFP on the membrane (same as × in the big graph) and its resolved two components.

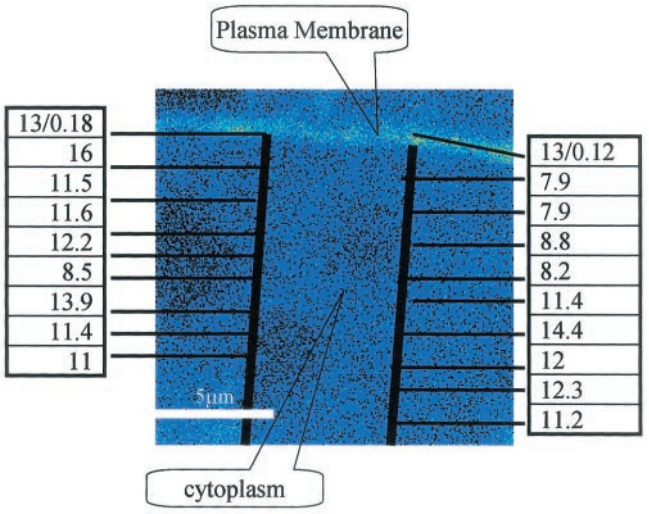

FIGURE 3 Map of the diffusion coefficients ($\mu m^2/s$) of AK1β-EGFP in the cell. The image shows a portion of a cell transfected with AK1β-EGFP. The bright line at the top of the image corresponds to the plasma membrane, and the lower segment of the image shows a portion of the cytoplasm. The numbers listed in the columns next to the images correspond to the diffusion coefficients ($\mu m^2/s$) measured at the indicated points across the cell. The first row in each column represents diffusion coefficients for the two components recovered from the autocorrelation curve at the membrane. At the plasma membrane, a dual diffusion rate was calculated from the FCS data. Away from the plasma membrane, single diffusion rates were found.

not surprising that the measured diffusion rates of our proteins varied among cells and different regions of the same cell. A useful way to represent the mobility of the protein in the cell would be to construct a spatial map of diffusion rates. Fig. 3 shows an example of such a diffusion rate map. As shown in the image, multiple points along two randomly selected lines were measured. The image, which is 15 μm in size, displayed only one-third of a cell in its *X-Y* plane. The full image of the cell is shown in Fig. 1 *B* (*middle panel*). The light green line on the upper part of the image corresponds to the plasma membrane. The cytoplasm of the cell is below the line. The numbers listed in the columns next to the images corresponds to the diffusion coefficients ($\mu m^2/s$) measured at the indicated points across the cell. All the autocorrelation curves were fitted with a single-component model unless the two-component model yielded a lower χ^2 value. The standard deviation of each fit is ~10% of its diffusion coefficient. The first row in each column represents diffusion coefficients for the two components recovered from the autocorrelation curves. This result was expected because the excitation beam was focused on the plasma membrane and surrounding areas. The other points of measurement do not involve the plasma membrane, and they can be fitted with a single-component autocorrelation function. The diffusion coefficients of AK1β-EGFP in the cytosol range from 7.9 to 16 $\mu m^2/s$, which strongly demonstrated that the dynamics of one kind of protein in the same cell could vary from region to region even though the fluorescence image appeared homogeneous. This result could be due to the differences in organelle fluidity, obstruction in diffusion, protein-protein interaction, or a temporal dependence to these parameters. A large sampling quantity is necessary to study the overall mobility of the chimeric proteins in the cell. Fig. 4 shows a composite histogram of the diffusion coefficients of the chimera proteins measured from dozens of cells and hundreds of dif-

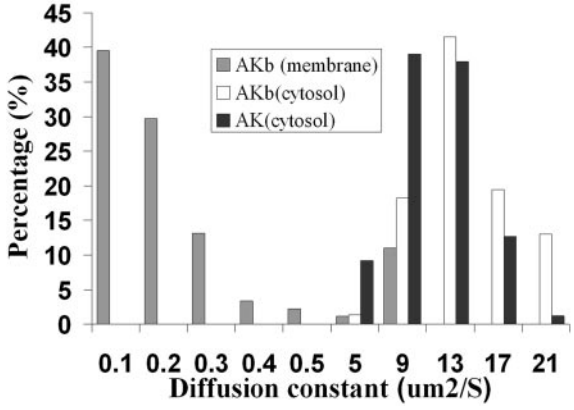

FIGURE 4 The composite histogram of the diffusion coefficients of EGFP fusion proteins in different cells and different regions of the cell. AK represents the distribution of diffusion coefficients (in percentage, the sum is 100%) of AK1-EGFP in HeLa cells. AKb (cytosol) represents the distribution of diffusion coefficients of cytosolic AK1β-EGFP. AKb (membrane) represents the distribution of diffusion coefficients of AK1β-EGFP on the plasma membrane of HeLa cells.

TABLE 1 Average diffusion coefficients of EGFP and EGFP fusion proteins in solution and in the cell

	Aqueous solution ($\mu m^2/s$)	Cytoplasm ($\mu m^2/s$)	Membrane ($\mu m^2/s$)
EGFP	87 ± 8.3	22.6 ± 3.7	
AK1-EGFP	61 ± 6.2	12.6 ± 3.4	
AK1β-EGFP		13.6 ± 4.8	0.23 ± 0.1

TABLE 2 Brightness and fluorescence lifetime of EGFP and EGFP fusion proteins

	Brightness (cpsm)	Lifetime (ns)
EGFP (solution)	4200 ± 600	2.80 ± 0.07
EGFP (cell)	4200 ± 800	2.45 ± 0.19
AK1-EGFP (cell)	3600 ± 600	2.34 ± 0.18
AK1β-EGFP (cell)	3600 ± 710	2.33 ± 0.28

ferent cell positions. The diffusion coefficient of AK1-EGFP and AK1β-EGFP measured inside the cell shared almost the same distribution with a peak around 13 $\mu m^2/s$ except that a small fraction of AK1β-EGFP had a slow component ($\sim$0.1–0.5 $\mu m^2/s$, not plotted in the graph). The slow component of AK1β-EGFP measured on the plasma membrane ranges from 0.1 to 0.5 $\mu m^2/s$ with a peak at 0.1 $\mu m^2/s$. The average diffusion coefficients of EGFP and its fusion protein are listed in Table 1. The standard deviation of the diffusion coefficient of EGFP (in solution and in the cell) was $\sim$10%, whereas the standard deviation of the diffusion coefficient of EGFP fusion proteins (in the cell) was 30–40%. The large standard deviation of EGFP fusion protein diffusion measured in the cell was not due to experimental error, but rather it reflected the dynamic complexity of AK1-EGFP and AK1β-EGFP in the cells.

PCH analysis

PCH analyses were performed on the same FCS data set. Because the brightness of a molecule is quadratically proportional to the input laser power on the sample, the laser power was kept constant (1 mW on the sample) for easy comparison of the brightness of the various proteins. All histograms could be fit with a single component. Fig. 5 shows an example of a PCH graph and the fitting of AK1β-EGFP measured in the cytosol of a HeLa cell. The molecular brightness of AK1-EGFP, AK1β-EGFP, and EGFP in HeLa cells are listed in Table 2. The fusion proteins have the same brightness, which is 3600 cpsm, although the corresponding fluorescence intensity is significantly different because the expression levels vary greatly. EGFP in the cell and in solution has a brightness of 4200 cpsm. The average number of EGFP or EGFP fusion protein molecules in the excitation volume ranged from 3 to 10 depending on the protein expression level in each particular cell. Based on the observation volume, their corresponding protein concentration was 30–100 nM.

Fluorescence lifetime imaging microscopy

The fluorescence lifetime of a fluorophore is a concentration-independent parameter that can be used to detect variations in the optical properties of a fluorophore or its environment. The fluorescence lifetime images of the EGFP, AK1-EGFP, and AK1β-EGFP in viable HeLa cells were measured and compared with EGFP in solution. In Fig. 6, the first column shows fluorescence intensity images of

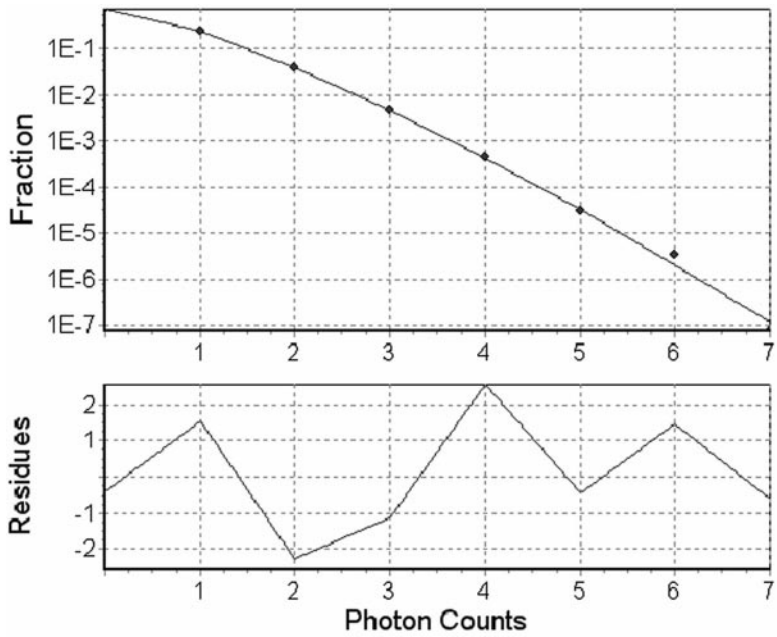

FIGURE 5 PCH of AK1β-EGFP measured in the cytosol of HeLa cell. The solid line represents a fit with a single species model. The lower panel shows the residues of the fit. The recovered number of molecules in the observation volume is 1.77, and the brightness is 3600 cpsm.

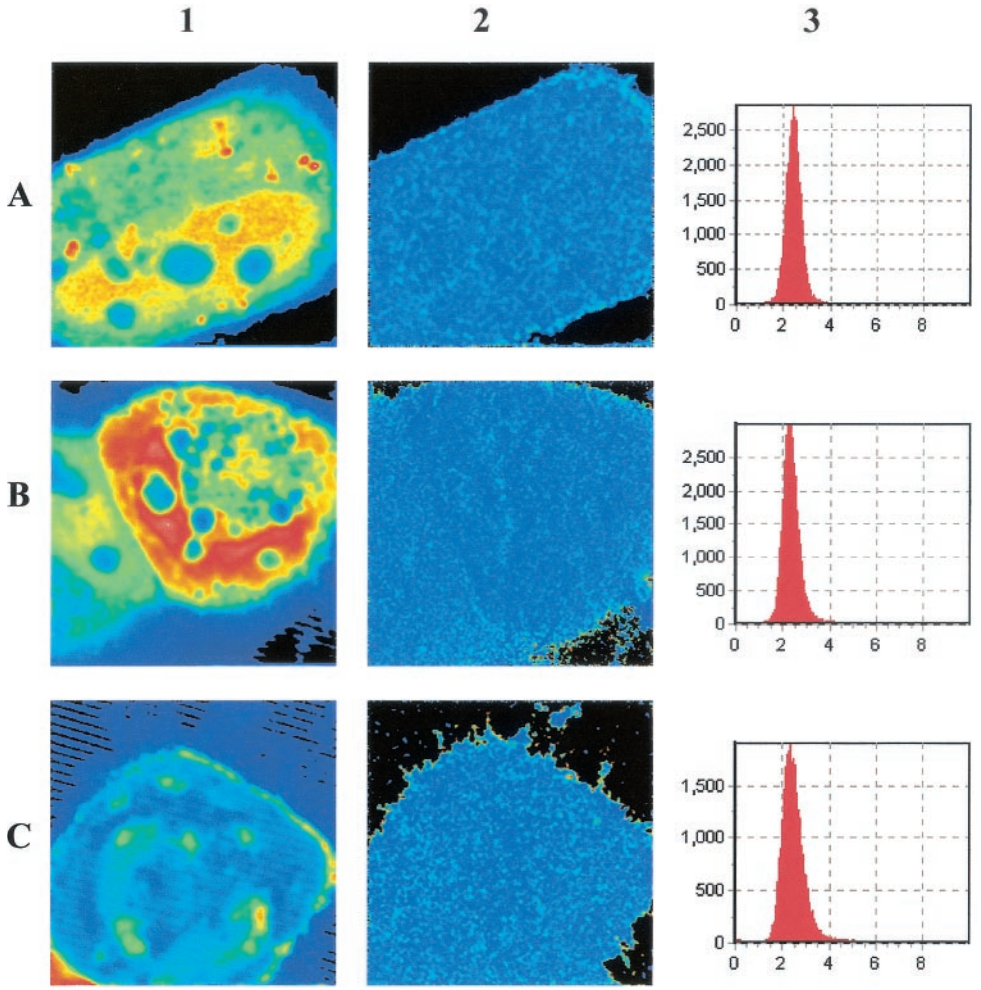

FIGURE 6 Fluorescence lifetime images of HeLa cells. (*A*) Images of EGFP-transfected cells; (*B*) Images of AK1-EGFP-transfected cells; (*C*) Images of AK1β-EGFP-transfected cells. Columns 1 and 2 show fluorescence intensity and lifetime images, respectively. Column 3 shows pixel lifetime histograms (abscissa in nanoseconds) corresponding to the images in column 2.

EGFP-, AK1-EGFP-, and AK1β-EGFP-transfected cells, respectively, the second column presents the corresponding fluorescence lifetime images, and the third column presents the pixel histogram of the lifetime images. From Fig. 6, it is clear that the lifetimes of EGFP, AK1-EGFP, and AK1β-EGFP were homogeneous throughout the cell. The average fluorescence lifetime of EGFP and its fusion protein in the cells are listed in Table 2. The lifetime of EGFP in solution (2.8 ns) was also measured, and it was longer than that in live cells (2.45 ns). The fluorescence intensity of the non-transfected cells was too low to allow for a fluorescence lifetime determination of the autofluorescence of the cell.

DISCUSSION

The main objective of this study was to characterize the localization of AK1 and AK1β in live cells and to demonstrate the applicability of FCS to quantitatively measure the dynamics of the target proteins in cellular environments.

Two-photon excitation is the simultaneous absorption of two photons by a molecule that is normally excited by a single photon with twice the energy (Friedrich, 1982). Two-photon excitation offers advantages over one-photon excitation in many aspects. For example, the excitation light can be easily separated from the fluorescence, so that only molecules at the microscope focus are excited, therefore reducing photo-damage to the sample outside the excitation volume, and the inherent optical sectioning effect is useful for fluorescence imaging and other microscopy measurements. The initial demonstration of two-photon FCS measurements in cells was reported by Berland et al. (1995). They investigated the diffusion characteristics of fluorescently labeled latex spheres inserted into fibroblast cells by electroporation. In recent years, a few reports on FCS applications in live cells have appeared (Gennerich and Schild, 2000; Kohler et al., 2000; Schwille et al., 1999a; Wachsmuth et al., 2000). FCS measurements are generally carried out at nanomolar or sub-micromolar sample concentrations, because the relative amplitude of the fluctuations is inversely proportional to the number of molecules simultaneously observed in the excitation volume. The upper limit for sample concentrations in FCS measurements is usually micromolar (Thompson, 1991). Sample concentration can be easily adjusted in solution; however, the cellular protein concentration is more difficult to adjust. The expression level

of a protein in the cell can be lowered by decreasing the plasmid concentration and linearizing the plasmid. Lowering the protein's expression level was also beneficial for studying the biological function of the protein in the cell, because it more closely mimics physiological conditions. Under optimal conditions, the concentration of the fusion protein in the cells was in the range of ~30–100 nM.

Expression and imaging of EGFP fusion protein

The fluorescence images of AK1-EGFP- and AK1β-EGFP-transfected cells (Fig. 1) revealed that AK1-EGFP is distributed throughout the cell except for some vesicles in the cytoplasm and the nuclear membrane. By contrast, AK1β-EGFP is mainly localized on the plasma membrane. Immunoblotting experiments carried out by Collavin et al. (1999) showed that AK1β was found in the plasma membrane fraction of the cell lysate. Our direct visualization of AK1β-EGFP in the cell confirmed their findings, which suggested that the AK1β protein was indeed targeted to the plasma membrane. In addition, we found that on average, the expression level of AK1-EGFP in the cell was much higher than that of AK1β-EGFP (Fig. 1), although the protein expression level varied from cell to cell. The cells with high AK1β-EGFP concentrations usually appeared unhealthy; they tended to round up or die. This observation suggests that AK1β might be toxic to the cells at higher concentration, whereas AK1 was not. The toxicity suggests that AK1β might have additional biological functions other than converting AMP and ATP to ADP. After all, the transcription level of the AK1β gene was shown to be upregulated by p53 (Collavin et al., 1999).

In the cytoplasm and nucleus of AK1β-EGFP-transfected cells, measurable levels of fluorescence were detected. Its origin could be from AK1β-EGFP or truncated EGFP. There have been reports showing that truncated GFP were found in transfected cells (Brock et al., 1999). A conventional method to discern AK1β-EGFP in the cytoplasm would be to run a Western blot to determine the presence of the fusion protein. The blot would require a specific antibody for the protein. With the application of FCS, the question can be easily resolved.

FCS and PCH analysis on EGFP fusion proteins

The fluorescence observed in the cytosol of an AK1β-EGFP-transfected cell could also arise from autofluorescence. Reduced pyridine nucleotide, flavin, and lipofuscin are the known fluorescent molecules, which contribute to the autofluorescence in mammalian cells (Andersson et al., 1998; Croce et al., 1999). Although the autofluorescence of the cell was low at 915-nm excitation, it was still possible to confuse it with the fluorescence from very low concentrations of AK1β-EGFP. FCS measurements carried out on nontransfected HeLa cells showed that there was no amplitude in the autocorrelation curve and low signal-to-noise ratio from the autofluorescence of the cell, suggesting that the autofluorescence came from many dim heterogeneous molecules. This observation was in agreement with reports from other laboratories (Schwille et al., 1999b; Wachsmuth et al., 2000). In the autocorrelation analysis, the contribution of each molecule is proportional to the square of its brightness. Because the brightness of the autofluorescence molecule is dim compared with EGFP, autofluorescence will contribute very little to the autocorrelation curve. FCS measurements carried out on the transfected yet dimly fluorescent cell showed robust autocorrelation curves with a high $G(0)$, indicating that the low fluorescence intensity was due to EGFP fusion protein expressed at low levels. The autocorrelation curves of cytosolic EGFP and AK1-EGFP can be fitted with a single component; no anomalous diffusion was observed.

Table 1 summarizes the averaged diffusion coefficients of EGFP and its fusion proteins in aqueous solution and in the cell. The diffusion coefficient of EGFP in the cytoplasm was approximately 3 times smaller than in solution. The ratio of D in the cytoplasm relative to that in solution ($D_{Cyt}/D_{H2}O$) is 0.26, which matches the Verkman group's report (Seksek et al., 1997). The cell lines and experimental conditions used to transfect EGFP and AK1-EGFP were the same. The size of AK1-EGFP is twice as large as EGFP. Since the diffusion coefficient of a particle should be proportional to the inverse cubic root relative to its size (assuming spherical shape), the calculated diffusion rate of EGFP should only be 25% faster than that of AK1-EGFP. However, in the cytoplasm, the $D_{EGFP}/D_{AK1\text{-}EGFP}$ is 1.8, which is 50% more than one would expect from free diffusion of AK1-EGFP. The unexpected slow diffusion rates of AK1-EGFP and AK1β-EGFP in the cell suggested that they were likely interacting with other proteins or forming oligomers in the cytoplasm. Our results also excluded the possibility of a truncated EGFP in the AK1-EGFP transfected cells.

FCS measurements not only confirmed the integrity of the fusion protein AK1-EGFP but also suggested the existence of some protein interactions. These interactions could be the oligomerization of the fusion proteins or the interactions with other cellular proteins. The PCH analysis provides information on the molecular brightness of the sample. For example, when a monomeric protein is singly labeled, then its dimer will appear twice as bright as the monomeric protein. Thus, by analyzing the molecular brightness of EGFP versus AK1-EGFP, we should be able to discern protein oligomerization by using molecular brightness analysis. The fact that the brightness of AK1-EGFP and AK1β-EGFP were roughly that of EGFP in the cell and in solution (Table 2) suggested that the fusion proteins didn't form oligomers in the cell. The slightly lower brightness in the fusion proteins, as compared with EGFP alone, suggested that the formation of a chimeric protein could slightly quench EGFP fluorescence. This observation of quenching was consistent with the change in

the measured fluorescence lifetime (Table 2). Therefore, the smaller diffusion coefficient of AK1-EGFP and AK1β-EGFP in the cell was not due to protein aggregation but rather interactions with other cellular structures or proteins. One candidate might be the potassium K_{ATP} channels. There was a report that AK communicates cellular energetic signals to ATP-sensitive potassium channels. Carrasco et al. (2001) found that the response of K_{ATP} channels to metabolic challenge was regulated by AK, and increased AK activity was detected in K_{ATP} channel immunoprecipitates. FCS measurements confirmed that the fluorescent particles in the cytoplasm and the nucleus of the AK1β-EGFP-transfected cell were AK1β-EGFP chimera proteins as well. A few rationales for its appearance in those regions include overexpression of the protein causing its nonspecific localization in the cells, the interaction between the protein and the membrane being not sufficiently strong to retain all proteins bound to the membrane, and AK1β protein being recruited to those regions for an unknown purpose.

Dynamics of AK1β-EGFP on the plasma membrane

Fluorescence images of AK1β-EGFP-transfected cells revealed that AK1β-EGFP was targeted to the plasma membrane of the cell (Fig. 1). Membrane proteins are classified as being either peripheral or integral. Nearly all known integral membrane proteins span the lipid bilayer. In contrast, peripheral proteins are bound to membranes primarily by electrostatic and hydrogen-bond interactions. Many peripheral membrane proteins are bound to the surfaces of integral proteins or are anchored to the lipid bilayer by a covalently attached hydrophobic chain, such as a fatty acid. FCS measurements of AK1β-EGFP in the membrane enabled us to ascertain the dynamic interactions between AK1β-EGFP and the membrane. For a variety of reasons, FCS measurements of membrane-bound protein are more challenging than those of cytoplasmic proteins. The diffusion rate of a membrane-bound protein is much slower than that of a cytoplasmic protein. The slow exchange rate of a protein in the laser focus tends to result in more photodamage to the membrane protein. The plasma membrane is thinner ($\sim$10 nm) than the height of the focal volume ($\sim$1 μm). If the fluorescent particles are present both on the plasma membrane and in the cytosol, when the laser beam is focused on the plasma membrane, it is not possible to isolate pure membrane diffusion from intracellular diffusion. One expects that the recovered autocorrelation curves should have multiple or distributed components. Analysis of our experimental results showed that the autocorrelation curves of AK1β-EGFP indeed have two components (Fig. 2 *inset*) when the laser was focused on the top of the cell (plasma membrane). The ratio of the amplitude of two components (fast:slow) is 1.2, and the ratio of the diffusion rates of the two component (fast:slow) is 70. We attributed the slow component to the lateral diffusion of AK1β-EGFP on the membrane, whereas the fast component corresponded to the diffusion of cytosolic AK1β-EGFP. By slightly changing the laser focal plane in the *Z* direction, the ratios of the amplitude contribution of the two components (fast:slow) were varied; they range between $\sim$0.39 and $\sim$6.8. In one data set, when focusing at the very top of the cell, we were able to measure 72% membrane-bound protein and 28% cytosolic protein in the excitation volume. Besides diffusion, there was also a possibility of an association/dissociation process of AK1β-EGFP between the membrane fraction and the cytosolic fraction, which could contribute to the fast component of the autocorrelation curve. The two components we observed were not due to anomalous diffusion on the plasma membrane. First, because changing the *Z*-direction focus affected the ratio of the two components in the autocorrelation curve. Second, the diffusion coefficient of the fast component matched that of cytoplasmic AK1β-EGFP. There have been reports that fluorescently labeled particles diffuse anomalously on cell membranes and inside the cell. However, EGFP in cells can be described by assuming a single diffusing species (Chen et al., 2002; Schwille et al., 1999b). It has been suggested that the anomalous diffusion of the fluorescent dye in cells may be due to interaction of the dye with intracellular structures or with much larger soluble molecules.

The lateral diffusion rates of cellular components on the membrane have traditionally been studied by fluorescence recovery after photobleaching (Jacobson et al., 1976). The reported diffusion rates of membrane proteins measured by fluorescence recovery after photobleaching range from 10^{-3} to 10^{0} $\mu m^2/s$, depending on their properties and location. For example, the diffusion coefficient for Na,K-ATPase is 3.3×10^{-2} $\mu m^2/s$ (Paller 1994), whereas the diffusion coefficient of the platelet-derived growth factor β-receptors in human fibroblasts can increase to 0.11 $\mu m^2/s$ upon the addition of platelet-derived growth factor (Ljungquist-Hoddelius et al., 1991). The diffusion coefficient of the slow component of AK1β-EGFP falls in the same range as that of other lipid-anchored proteins (Niv et al., 1999). This observation suggested that some of the AK1β-EGFPs were indeed bound to the membrane. Because AK1β-EGFP and AK1-EGFP differ only by the 18 amino acid residues at the N-terminus, and AK1-EGFP doesn't bind to the plasma membrane, it is likely that the 18 amino acid residues are interacting with the inner surface of the plasma membrane. Alternatively, these residues provide a signal for protein modification that enables the protein to bind to membrane or a combination of both possibilities. Dr. Schneider's group, discoverers of this novel gene first suggested the possibility of lipid modification on the second residue of the AK1β (Collavin et al., 1999). Our in vitro studies of AK1β confirmed that AK1β has a signal for protein myristoylation, and the fatty acid chain enabled its binding to the lipid bilayer. The results of the in vitro characterization of AK1β will be presented in another study.

We observed unevenly distributed fluorescence intensity on the plasma membrane based on the fluorescence images of the AK1β-EGFP-transfected cells (Fig. 1), suggesting the possibility of some AK1β-EGFP membrane clustering. Our PCH analysis showed that these clusters were not AK1β-EGFP aggregates but rather represented a membrane region with a higher AK1β-EGFP concentration. The plasma membrane presents an intriguing mix of dynamic process activities in which components may randomly diffuse, be confined transiently to small domains (lipid raft), or experience highly directed movements (Jacobson et al., 1995). The diffusion coefficient of AK1β-EGFP we measured on the membrane ranges between $\sim$0.1 and $\sim$0.5 $\mu m^2/s$ (Fig. 4). The wide distribution in diffusion coefficient could be due to the complexity of the membrane organization.

Fluorescence lifetime imaging and molecular brightness

The fluorescence lifetime of a probe is a concentration-independent parameter, which has been widely used to study molecular interactions in the fluorescence resonance energy transfer method. The fluorescence lifetime of EGFP in solution was reported to be 2.9 ns and 2.6 ns in the cytosol (Swaminathan et al., 1997). We are interested in examining the effect of the fusion protein on the lifetime of EGFP and comparing the results with our PCH analysis. The fluorescence lifetime of EGFP in solution (pH 7.2) was 2.8 ns, which agrees with the reported value. The lifetime of EGFP measured in the cell was 2.45 ns, which was shorter than the value measured in solution. The lifetime of EGFP fusion proteins was slightly smaller than that of EGFP in the cell, which was probably due to quenching. These results appeared to be somewhat contradictory with the brightness of EGFP obtained from PCH analysis, where EGFP in solution and in the cell has identical brightness. The difference in lifetime (2.8 ns vs. 2.45 ns) could be due to the cellular environment. We have considered two factors: the pH and the background fluorescence. Control experiments showed that the lifetime of EGFP was insensitive to pH variation in the range of pH 5–9. Therefore, the pH difference between the solution and the intracellular environment probably was not the contributing factor. The background fluorescence and scattering were the most likely contributing factors in the variation of lifetime in the cell. Molecules that produced background fluorescence and the scattering light tend to have an apparent short lifetime, which could shorten the average lifetime even though its fluorescence intensity contributes less than 10% of the total fluorescence intensity measured in the transfected cell. In the PCH analysis, the brightness of background fluorescence molecule is much lower than that of EGFP (Chen et al., 2002; Schwille et al., 1999b); therefore, it has little effect on the brightness of EGFP and its fusion proteins in the cell. We believe the brightness of EGFP measured in the cell better represents its fluorescence property than the apparent lifetime.

SUMMARY

In summary, we constructed and expressed chimeric proteins of EGFP and AK isoforms (AK1 and AK1β) in HeLa cells. We directly visualized the distribution of two related fusion proteins in viable cells: AK1-EGFP was distributed throughout the cell and AK1β-EGFP was mostly localized on the plasma membrane. We quantitatively measured the diffusion coefficients of the fusion proteins in different regions of the cell. The diffusion of EGFP and AK1-EGFP in the cytoplasm was described by assigning a single diffusing species, and no anomalous diffusion was observed. There was some AK1β-EGFP distributed in the cytoplasm and nucleus of the cell, including a portion that may interact with intracellular membranes or other structures. AK1β-EGFP showed a similar diffusion rate distribution as AK1-EGFP, suggesting that their interactions with the intracellular environment are similar. Multiple diffusion species were recovered from the autocorrelation curves of AK1β-EGFP measured on the plasma membrane. Detection of the slow-diffusion component suggested that AK1β-EGFP was bound to the plasma membrane and underwent lateral diffusion on the membrane. The fast-diffusing species could arise from the cytosolic AK1β-EGFP or the exchange of AK1β-EGFP between its cytosolic and membrane-bound fractions. The brightness of EGFP and its fusion proteins suggested that they do not form aggregates in the cell. The apparent fluorescence lifetime of EGFP and its fusion protein in the cell was shorter than that of EGFP in solution. This could due to the contribution of background fluorescence. Our system (AK1-EGFP and AK1β-EGFP) provides a good example of studying isozyme properties in the cell using fluorescence microscopy. We particularly note the ability to distinguish different localization and diffusion rates, in turn reflecting functional differences. Of course, these techniques can also be extended to study intrinsic protein interactions or those with drugs in live cells.

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