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Site-Directed Mutagenesis Enabled Preparation of a Functional Fluorescent Analog of Profilin: Biochemical Characterization and Localization in Living Cells

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The preparation of fluorescent profilin analogs for binding and spectroscopic studies, in vitro and in vivo, has been hampered by the poor chemical reactivity of this protein in its native form. We have addressed this problem by labeling a mutant, chemically reactive form of profilin. Site-directed mutagenesis was first used to replace a serine residue in a non-essential domain with a reactive cysteine residue. The mutant protein was expressed in *Escherichia coli* and reacted with tetramethylrhodamine iodoacetamide. In vitro assays indicated that the fluorescent profilin maintained its ability to bind actin, polyproline, and PIP_2, to inhibit actin polymerization, and to stimulate actin nucleotide exchange. Fluorescence spectroscopy showed that neither the excitation nor the emission of the analog was sensitive to the interaction with actin or polyproline. However, binding of PIP_2 caused a 75% quenching of the fluorescent signal, suggesting a dramatic change in the immediate environment of the probe. When the fluorescent profilin was microinjected into living NRK cells, it became localized at cell-cell junctions and discrete sites near the anterior end, where it colocalized with aggregates of unpolymerized actin. Different engineered forms of profilin with fluorophores located at defined sites should greatly facilitate the study of its interactions with various ligands and cellular structures.

Key words: fluorescence spectroscopy, microinjection, actin assembly

INTRODUCTION

Profilin, a 12–15 kd protein, was the first identified intracellular protein capable of sequestering actin monomers and inhibiting the assembly of actin filaments [Carlsson et al., 1977]. Investigations during the past 20 years have provided detailed information about this molecule from gene sequence and atomic structure to potential cellular function [Haarer and Brown, 1990; Haarer et al., 1990; Cooley et al., 1992; Theriot and Mitchison, 1993; Metzler et al., 1993; Cedergren-Zeppezauer et al., 1994; Balasubramanian et al., 1994]. Besides the association with actin, profilin can bind phosphoinositides and polyproline in vitro [Lassing and Lindberg, 1985, 1988; Tanaka and Shibata, 1985; Archer et al., 1994]. The association with phosphatidylinositol 4,5-diphosphate (PIP_2) has been proposed as a possible mechanism for the regulation of its actin-binding activities [Machesky and Pollard, 1993; Sohn and Goldschmidt-Clermont, 1994].

Despite these studies, the physiological role of profilin and of its various binding interactions is still poorly understood. While a number of cellular studies are consistent with the ability of profilin to sequester actin molecules [Cao et al., 1992; Haugwitz et al., 1994], other

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observations suggest that profilin can promote actin assembly under certain conditions [Mockrin and Korn, 1980; Goldschmidt-Clermont et al., 1992; Pantaloni and Carlier, 1993; Finkel et al., 1994]. Moreover, it is controversial as to the role of profilin in the assembly of actin filaments at the tail of invading *Listeria* [Theriot et al., 1994; Marchand et al., 1995]. Besides the regulation of actin assembly, profilin may have additional functions in signal transduction pathways involving cAMP [Vojtek et al., 1991; Goldschmidt-Clermont and Janmey, 1991], or growth factor-activated phospholipase C [Goldschmidt-Clermont et al., 1990, 1991a]. Further, the ability of profilin to bind polyproline suggests that it may interact with a number of signaling molecules that contain proline-rich domains [Williamson, 1994].

An important step toward understanding the function and regulation of profilin is to define the interaction of profilin with various ligands, cell membranes, and actin-containing structures in vitro and in living cells. So far only limited information is available concerning the distribution of profilin [Hartwig et al., 1989; Goldschmidt-Clermont et al., 1991b; Buß et al., 1992a; Machesky et al., 1994], partially due to limitations in immunocytochemical techniques. In order to allow direct analyses of the dynamics of profilin and its mutants in living cells, it will be useful to apply the approach of fluorescent analog cytochemistry [Wang, 1992], where profilin localization can be observed directly with fluorescent analogs microinjected into living cells. In addition, spectroscopic characteristics of such probes may also be used as a powerful means for revealing conformational changes or ligand binding.

Our initial efforts to produce fluorescent profilin were hampered by the low reactivity of wild-type mammalian profilin toward common fluorescent reagents. In the present study, we have taken an alternative approach, where a functional, reactive form of profilin was generated by site-directed mutagenesis. The mutant protein was subsequently expressed in *E. coli* and successfully labeled with a fluorophore. In vitro assays indicate that the fluorescent profilin analog retained most if not all of its functional activities. Surprisingly, the fluorescence intensity was dramatically quenched in PIP_2 but not by actin or polyproline, suggesting that the labeled site was in the vicinity of a PIP_2 binding site. Microinjection of this probe into living cells resulted in the incorporation of the fluorescent analog into G-actin-rich punctate sites behind the lamellipodia and regions of cell-cell adhesion.

MATERIALS AND METHODS

Preparation of Mutant Profilin

All restriction enzymes were purchased from New England Biolabs (Beverly, MA). A clone of rat profilin cDNA was kindly provided by Dr. Peter Rubenstein (University of Iowa, Iowa City, IA). A Nco I-Sal I fragment containing the complete coding region for profilin was then cloned into the NcoI-Xho I site of the expression vector pET 14b under the control of T7-inducible promoter (Novagen, Madison, WI). This expression vector, referred to as pET14-prof, was transformed into *E. coli* strain BL-21 (DE3) lysogenic for T7 polymerase. The entire coding region of profilin in the expression construct was sequenced to confirm that there was no mutation as compared to the genebank sequence, using sequencing kit (Sequenase 2.0, U.S. Biochemicals, Cleveland, OH) and adenosine 5′-(α-thio) triphosphate S^{35} from New England Nuclear (Boston, MA). Profilin expression was induced with isopropyl thiogalactoside (dioxane free, U.S. Biochemicals) following standard protocols [Studier et al., 1990]. Profilin was affinity purified from homogenized bacterial suspension using polyproline affinity chromatography (Sigma, St. Louis, MO) according to Kaiser et al. [Kaiser et al., 1989].

The wild type clone was engineered to replace Serine 41 with cysteine by PCR mediated site directed mutagenesis using the Vent polymerase (New England Biolabs). A mutant primer TCC CCG GGA AAA CCT TCG TAT GCA TTA CGC C and a T7 terminator primer GCT CAG CGG TGG CAG CAG CC (synthesized by Integrated DNA Technologies, Coralville, IA) were used in the PCR reaction with the complete plasmid as template to generate a mutant fragment of the profilin gene. The primer mediated three changes, a point mutation from Serine (AGC) to cysteine (TGC), improved codon usage for lysine from AAG to AAA, and the creation of a Nsi I site (ATGCAT) to facilitate screening of mutant clones. The PCR product was purified with QIA quickspin PCR Purification kit (Qiagen, Chatsworth, CA), cut with Bam H I and Sma I, subcloned into the Bam H I-Sma I site of pET14-prof, and transformed into XL-1-Blue. The appropriate clone was selected by the presence of a Nsi I site and then sequenced to confirm the mutation. The plasmid was purified and transformed into the BL-21 host. Mutant profilin was expressed and isolated with the protocol identical to that used for the wild type profilin [Kaiser et al., 1989].

Fluorescent Labeling of Profilin

Profilin was labeled with either the mixed isomer or isolated 5-isomer of tetramethylrhodamine iodoacetamide (Molecular Probes, Eugene, OR). Briefly, purified profilin at a concentration of 10–15 mg/ml was treated with 10 mM dithiothreitol (DTT) and dialyzed overnight against 50 mM borate buffer, pH 8.4. Tenfold molar excess of the dye was first dissolved in acetone at a concentration of 1.5 mg/ml, then mixed with the borate buffer to obtain a concentration of 0.8 mg/ml. The dye

solution was clarified at 100,000*g* for 10 min. and mixed with an equal volume of profilin under an N_2 atmosphere. The final concentration of acetone is 25%. The mixture was centrifuged at 100,000*g*, room temperature for 2 h during which the reaction took place. The supernatant was then passed through a Bio-Beads SM-2 column (Bio-Rad, Richmond, CA) to remove unreacted dye. The eluant was then concentrated and stored under liquid N_2. Labeled profilin was examined with 15% SDS-PAGE [Laemmli, 1970] to ensure the absence of free dye. The concentration of profilin was measured by the method of Lowry et al. [1951], and the concentration of associated tetramethylrhodamine was estimated based on a molar extinction coefficient of 60,000 at 555 nm.

Binding Assays of Labeled Profilin

The ability of fluorescent profilin and unlabeled wild type profilin to bind polyproline was compared by a batch adsorption method. A 50 μl suspension of polyproline-Sepharose in 100 mM NaCl, 100 mM glycine, 1 mM DTT, 10 mM Tris-Cl, pH 7.8, was mixed with 80 μl of 1.0 mg/ml profilin in the same buffer. The mixture was incubated for 30 min on ice, and the sepharose gel was then pelleted by centrifugation at 500*g* for 5 min in an Eppendorf 5415 microcentrifuge (Madison, WI). The concentration of unbound protein in the supernatant was measured with the Lowry assay. The range of total unbound profilin was calculated assuming that Sepharose accounts for either 0 or 50 μl of the total volume.

PIP_2 binding was measured following a modified procedure of Haarer et al. [1993]. Profilin in a buffer of 75 mM KCl, 0.5 mM DTT, 10 mM Tris-HCl, pH 7.4, was mixed with PIP_2 micelles, prepared by sonicating a freshly thawed aliquot of about 900 μM PIP_2 (1 mg/ml; Sigma) in water, to obtain a final concentration of 4 μM profilin and 300 μM of PIP_2. Samples of 400 μl were incubated on ice for 30 min and then layered onto Millipore (Bedford, MA) PLTK filters (30 kd cut off) and spun at 2,000*g* in an Eppendorf microcentrifuge for 10 min. The filtrates from samples with or without PIP_2, together with the loaded samples, were analyzed with SDS-PAGE.

Measurement of actin binding was also done with PLTK filters. Profilin and purified muscle actin were first dialyzed into a buffer of 0.5 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 2 mM Tris-HCl, pH 8.0. The two were then mixed to obtain a final concentration of profilin at 13 μM and actin at 18 μM. The mixture was incubated on ice for 30 min. Samples of 400 μl were then layered onto Millipore PLTK filters and spun as described above. The percentage of profilin associated with actin was calculated based on the amount of profilin that passed through the filter in the presence or absence of actin.

Functional Assays of Labeled Profilin

The rate of actin nucleotide exchange was measured according to Goldschmidt-Clermont et al. [1991b]. Briefly, at zero time 50 μM of 1,N^6-ethenoadenosine 5′-triphosphate (εATP) was added to 0.6 μM column purified muscle actin in 2 mM Tris, 1 μM $CaCl_2$, 2 μM ATP, 5 μM DTT, pH 7.2, with profilin at 0, 0.12 or 0.24 μM. The fluorescence intensity was measured in a Perkin Elmer (Oak Brook, IL) LS-3 fluorimeter with excitation wavelength of 360 nm and emission wavelength at 410 nm. The fluorescence intensity of the zero time reading was subtracted from all other time points. Profilin alone, in the absence of actin, did not alter the fluorescence intensity of εATP.

The effect of profilin on actin polymerization was measured by the pyrene actin assay [Kouyama and Mihashi, 1981; Pantaloni and Carlier, 1993]. A 1 μM solution of actin containing 10% pyrene actin was mixed with 0, 1, 2, 5, 10, 15, 20, 25 μM profilin. The fluorescence intensity was measured in a Perkin Elmer LS-3 fluorimeter with excitation wavelength of 365 nm and emission wavelength at 407 nm. Polymerization was induced with the addition of 0.1 M KCl and 2 mM $MgCl_2$. After 24 h the fluorescence intensity was measured and corrected for the zero time reading.

Fluorescence Spectroscopy

Fluorescence excitation and emission spectra were measured in a computer-driven spectrofluorimeter (Photon Technology Instruments, Alphascan II, Santa Clara, CA). A reference channel in the instrument corrects temporal and spectral variabilities of the excitation light. The bandpass for both the excitation and emission monochromators was set at 2 nm. Excitation spectra were measured by varying the excitation wavelength from 450 to 590 nm in 0.25 nm increments with the emission wavelength set at 610 nm. Emission spectra were measured from 530 to 630 nm in 0.25 nm increments with the excitation wavelength set at 510 nm. All samples contained 5 μM profilin in a buffer of 0.5 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM DTT, 2 mM Tris-HCl, pH 8.0. Samples of profilin complexed actin, polyproline, and PIP_2 were prepared by incubating 5 μM profilin with a 10-fold molar excess of the ligand for 90 min before dilution.

Culture and Handling of Cells

A flat subclone of Normal Rat Kidney cells (NRK Strain 52E, ATCC, Rockville, MD) was grown in F12K medium supplemented with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS) [Fishkind and Wang,

1993], and was used for microinjection 36–48 h after plating [Cao et al., 1992]. Cells were maintained on a custom designed microscope stage incubator with CO_2 and temperature controls. The injection solution contained a mixture of 1.5–2.0 mg/ml rhodamine profilin and 4 mg/ml rhodamine green dextran in 5 mM Tris acetate, pH 7.0 (rhodamine green dextran 10 kDa; Molecular Probes catalogue D7153. The fluorescence characteristics of rhodamine green is similar to fluorescein). Most injections were performed with cells located around the periphery of a colony. The pressure for microinjection was regulated with a precision electronic pressure regulator. Approximately 3–5% cell volume was microinjected into each cell. Cells were subsequently incubated for 1–2 h to reach a steady state and then observed for up to 3 h.

Cells were fixed with 4% formaldehyde and extracted with acetone at −20°C as described previously [Cao et al., 1992]. Direct staining of unpolymerized actin with vitamin D-binding protein was performed following the procedure of Cao et al. [1993].

Endogenous profilin concentration was measured by Western blotting [Towbin and Gordon, 1984] of whole cell lysates with a polyclonal antibody generated against wild type profilin (East Acres Biologicals, Southbridge, MA). The bands on nitrocellulose were developed with the alkaline phosphatase reaction and were quantified by densitometry. The density of profilin bands was compared to that of standards containing known amounts of purified profilin.

Microscopy and Image Processing

All observations were performed with a Zeiss IM35 or Axiovert inverted microscope and a 100×/NA 1.30 Neofluar, a 63×/NA 1.25 Neofluar, a 40×/NA 1.0 Apochromatic, or a 40× Achromatic phase objective lens. A 100 W quartz-halogen lamp was used as the light source for epi-illumination. Fluorescence images were acquired with a cooled CCD camera (Princeton Instruments, Trenton, NJ) and processed for background subtraction. Image acquisition, processing, and ratio imaging were done with programs developed in house. Before the calculation of ratio images, each of the two images was filtered to set the intensity of the background to zero, without changing other pixels. The ratio value was set to zero if the pixel value in either image was zero. Confocal images of fixed cells were obtained with a Bio-Rad MRC-1000 scanning laser confocal system attached to a Nikon Diaphot microscope with a 63×/NA 1.4 Planapo objective lens. Some images were contrast enhanced before the preparation of prints. Prints were prepared with either the standard photographic procedure or a dye sublimation printer (Kodak ColorEase PS printer).

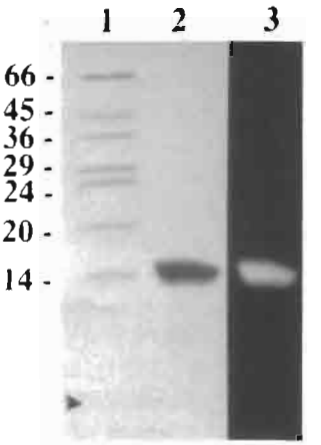

Fig. 1. Fluorescent labeling of mutant profilin. Labeled mutant profilin was analyzed with 15% SDS-PAGE. **Lane 1,** molecular weight markers. **Lane 2,** mutant profilin, purified and fluorescently labeled as described in Materials and Methods, stained with Coomassie blue. **Lane 3,** fluorescence of labeled mutant profilin, recorded with UV illumination before Coomassie blue staining. The arrowhead near the bottom marks the position of the tracking dye.

RESULTS

Construction of a Reactive Mutant Form of Profilin

We first attempted to fluorescently label wild type profilin prepared from either bovine brain or from *E. coli* transfected with a vector containing a rat profilin gene. Using a number of rhodamine-based fluorescent reagents under various conditions, we obtained only products either low in the extent of labeling or heavily contaminated with non-covalently associated dyes.

To facilitate the preparation of fluorescent conjugates, we constructed a plasmid that encoded a mutant form of profilin with a reactive sulfhydryl group. Our choice for the site of point mutation was based on several criteria. It should be located outside known binding sites or main secondary structures, should be relatively unconserved through evolution, and should have a hydrophobicity similar to that of cysteine. Serine 41 was identified as one of such sites. Point mutation from its AGC codon to TGC for cysteine, together with a new restriction site to facilitate screening, was created with PCR using a mutant primer. The plasmid expressed high levels of the mutant profilin upon transformation into *E. coli* strain BL-21 and IPTG induction. In addition, the protocol previously used for purifying bacterially expressed wild type profilin, based on affinity binding in a polyproline column, functioned equally well for the mutant profilin (Fig. 1).

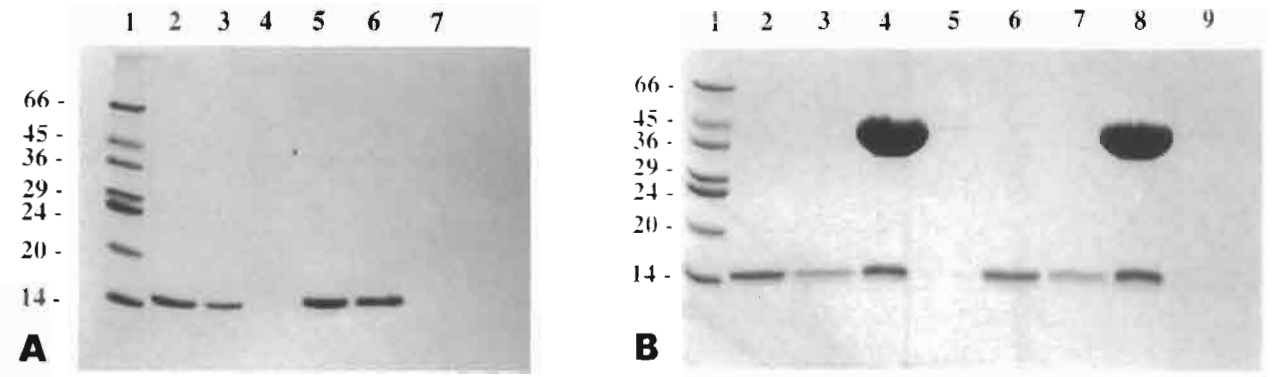

Fig. 2. Binding of wild type and labeled mutant profilin to PIP_2 or actin. Profilin was mixed with PIP_2 (A) or actin (B) and loaded onto PLTK filters as described in Materials and Methods. The load mixtures and the filtrates from wild type and labeled mutant profilin were analyzed with 15% SDS-PAGE. Since only unbound profilin was able to pass through the filter, the absence of profilin from a lane reflects its ability to bind PIP_2 or actin (B). Controls were performed with profilin loaded alone. **A: Lane 1,** molecular weight markers. **Lane 2,** wild type profilin-PIP_2 mixture before passing through the filter. **Lane 3,** filtrate from wild type profilin in the absence of PIP_2. **Lane 4,** filtrate from the mixture of wild type profilin and PIP_2. **Lane 5,** labeled profilin-PIP_2 mixture before passing through the filter. **Lane 6,** filtrate from labeled profilin in the absence of PIP_2. **Lane 7,** filtrate from the mixture of labeled profilin and PIP_2. **B: Lane 1,** molecular weight markers. **Lane 2,** wild type profilin in the absence of actin, before passing through the filter. **Lane 3,** filtrate from the sample as shown in lane 2. **Lane 4,** actin-wild type profilin mixture before passing through the filter. **Lane 5,** filtrate from the sample as shown in lane 4. **Lane 6,** labeled profilin in the absence of actin before passing through the filter. **Lane 7,** filtrate from the sample as shown in lane 6. **Lane 8,** actin-labeled profilin mixture before passing through the filter. **Lane 9,** filtrate from the sample as shown in lane 8. The presence of very faint bands of profilin and actin in lanes 5 and 9 reflects a slight leakage through the filter.

Preparation and Characterization of Fluorescent Profilin

As shown in Figure 1, the mutant profilin reacted successfully with either isolated 5-isomer or the 5- and 6-mixed isomers of tetramethylrhodamine iodoacetamide. The dye to protein ratio of fluorescent conjugation was typically 0.8 and 1.0, with no detectable non-covalently associated dye (Fig. 1). Identical reaction conditions, when applied to the wild type profilin, yielded an undetectable level of conjugation. Thus the fluorophore was most likely located at the engineered Cys 41 site.

Assays were performed to assess the ability of the fluorescent profilin to bind known ligands. In a batch assay, both wild type and labeled profilins bound to polyproline at a level higher than 95%. The binding of PIP_2 and actin was assayed with PLTK filters, which retained ligand-bound profilin but allowed free profilin to pass through [Haarer et al., 1993]. As shown in Figure 2A, no protein could be detected in the filtrate of samples containing PIP_2 and either wild type or labeled profilin. The association with actin was also comparable between wild type and labeled profilins (Fig. 2B). Under our assay conditions, about 74% of wild type profilin was bound to actin as compared to 69% of mutant labeled profilin, yielding an apparent K_d of 4.0 μM for labeled mutant profilin and 2.8 μM for unlabeled wild type profilin.

The ability of labeled profilin to stimulate actin nucleotide exchanges was assayed with ϵATP, a fluorescent analog of ATP. Exchange of excess ϵATP with actin-bound ATP/ADP manifested as an increase in fluorescence intensity, due to the sensitivity of ϵATP to actin binding. As shown in Figure 3, wild type and labeled mutant profilin caused a similar acceleration in the increase of fluorescence intensity, indicating that labeled profilin was indistinguishable from the wild type in terms of its ability to stimulate actin nucleotide exchange. The ability of labeled profilin to inhibit actin polymerization was assayed by measuring the extent of steady state actin polymerization with a trace amount of pyrene-actin in the presence of increasing concentrations of profilin (Fig. 4) [Pantaloni and Carlier, 1993]. Based on the X intercept and a critical concentration of 0.1 μM, we obtained an apparent K_d for the labeled mutant profilin at 3.82 μM, and an apparent K_d of 5.08 μM for the unlabeled wild type profilin.

The labeled profilin showed an excitation peak wavelength at 549.25 nm and an emission peak wavelength at 574.00 nm. Neither actin nor polyproline has an appreciable effect on the peak wavelength or intensity of the spectra (Fig. 5A,B). However, binding to PIP_2 caused a 75% decrease in excitation and emission fluorescence intensity, without a significant change in the peak excitation or emission wavelength (Fig. 5A,B).

Microinjection of Fluorescently Labeled Profilin Into Living Epithelial Cells

Rhodamine labeled profilin was microinjected into NRK cells at a concentration of 1.5–2.0 mg/ml. Based

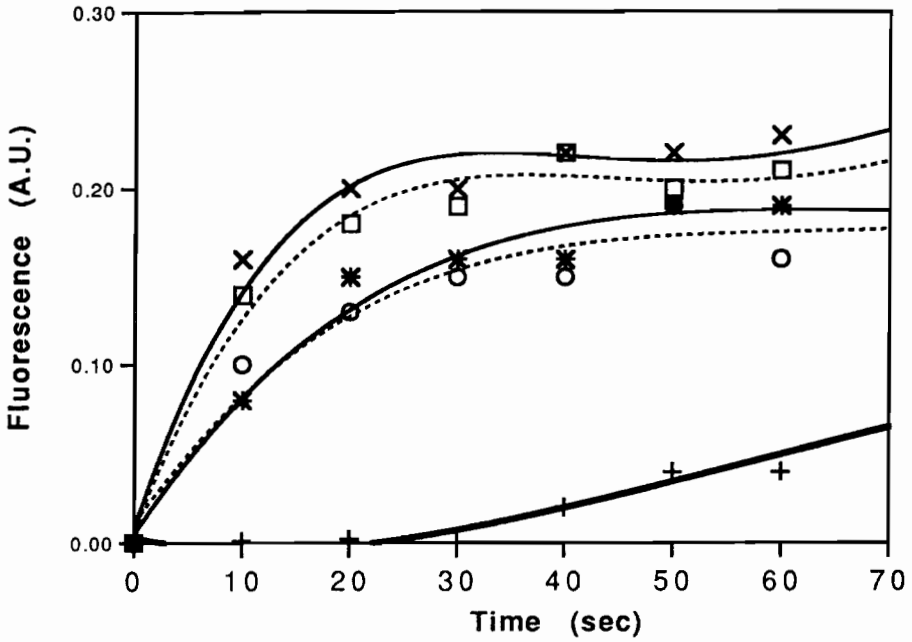

Fig. 3. Effects of wild type and labeled profilin on the rate of actin nucleotide exchange. Actin-ϵATP mixtures were mixed with 0.12 (*, ○) or 0.24 μM (x, □) of wild type (*, x, solid line) or labeled mutant profilin (○, □, dotted line). Actin in the absence of profilin was used as the control (+, dark line). Readings were taken every 10 sec and plotted as arbitrary fluorescence units. Exchange of ϵATP with actin bound ATP causes an increase in the fluorescence intensity. Lines through the points were fit by fourth order polynomial.

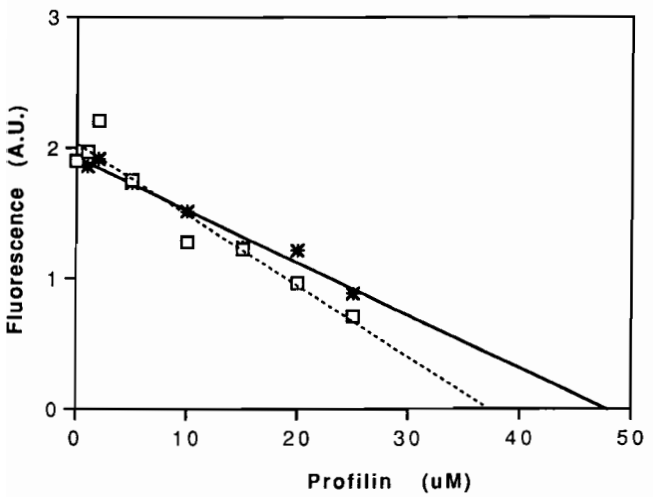

Fig. 4. Effect of wild type and labeled profilin on steady state actin polymerization. G-actin (1 μM) containing 10% pyrene actin were incubated with various concentrations of wild type (*, solid line) or labeled (□, dotted line) profilin and allowed to polymerize for 24 h. The y-axis represents the increase in fluorescence over that at time zero. Both wild type and labeled profilin cause a decrease in the extent of actin polymerization. The r values for the regression lines were 0.978 and 0.963, respectively. The affinity for profilin-actin binding was determined based on the X-intercept. Lines through the data points were created by linear regression.

on a cellular profilin concentration of 40 μM, as estimated by quantitative Western blotting (data not shown), the injection should cause an increase in profilin concentration by 8–17%. This increase was much smaller than that in previous "overexpression" experiments by Cao et al. [1992], and caused no noticeable effect on cellular motile activities or stress fiber organization. To provide a reference for the accessible volume in the cell, an inert low molecular weight green fluorescent dextran was microinjected along with profilin. Ratio imaging of the two images identified regions that were preferentially enriched or depleted with profilin.

Profilin in live cells appeared to distribute preferentially at cell-cell adhesion areas (Fig. 6d,f,g,i; n = 77 cells). In addition, in cells with a defined anterior-posterior polarity, ratio images of profilin to dextran revealed a discrete region of high profilin fluorescence towards the anterior of the cell (Fig. 6c, f; n = 33 cells). The perinuclear region showed a high intensity of profilin (Fig. 6a,d), apparently due to the increased depth of the cell since even dextran showed intense fluorescence in this region (Fig. 6b,e). Interestingly, the nuclear region had a lower intensity relative to the perinuclear region, (Fig. 6a,c,d,f; n = 160 cells), whereas dextran showed a much higher intensity in the nucleus (Fig. 6b,e).

With a confocal laser scanning microscope, prominent foci and some fibril-like structures can be found within and directly behind the lamellipodia (Fig. 7c; n = 151 cells). Since unpolymerized actin has been found to concentrate at punctate structures in a similar region [Cao et al., 1993], we wanted to determine whether these

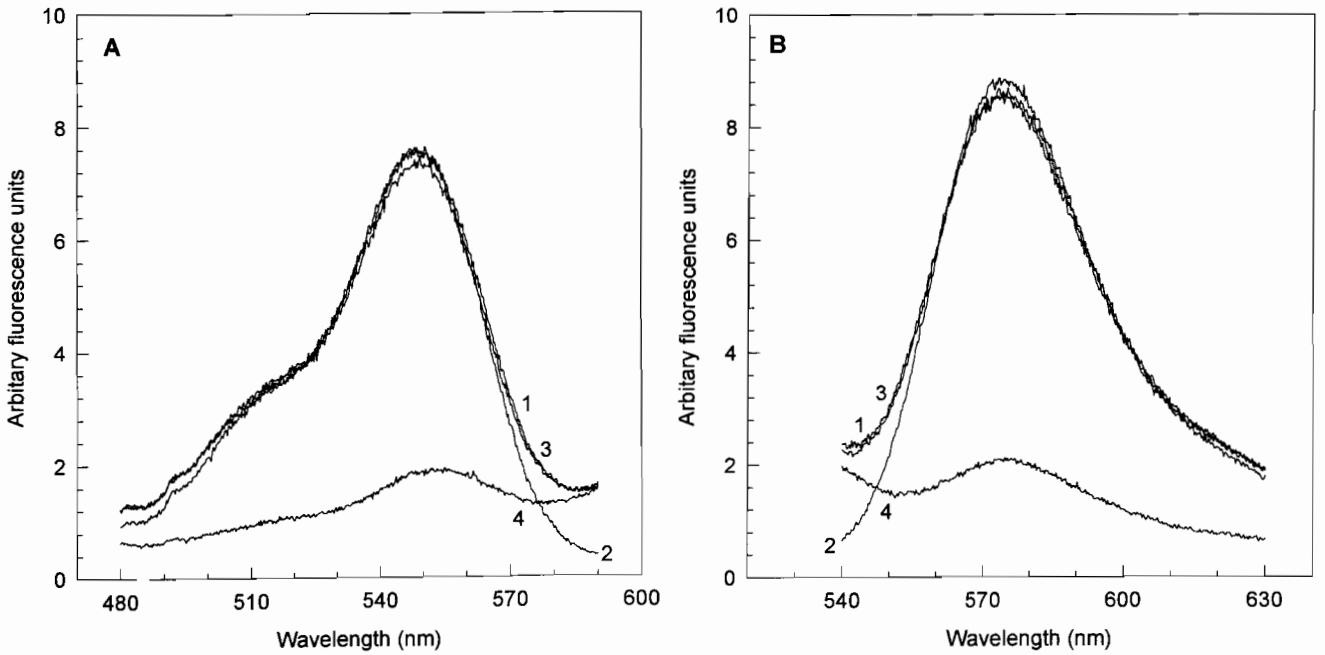

Fig. 5. Fluorescence spectra of labeled mutant profilin and its complexes with different ligands. The profilin was labeled with the 5-isomer of tetramethylrhodamine iodoacetamide. Excitation (**A**) and emission (**B**) spectra were recorded for 5 μM profilin (1), or complexes of profilin with 10-fold molar excess of actin (2), polyproline (3), and PIP_2 (4).

structures colocalize with the profilin-rich foci. Figure 7 shows cells microinjected with rhodamine profilin and subsequently stained with fluorescein vitamin D-binding protein, which serves as a specific label for unpolymerized actin [Cao et al., 1993]. Most of the G-actin-rich structures were also labeled with profilin (Fig. 7a, 7b; n = 63 cells), suggesting that at least some of the unpolymerized actin subunits within these structures were associated with profilin. Time-lapse observations of live cells indicated that the profilin-rich foci were transient, highly dynamic structures (n = 99 cells, data not shown) showing active changes concomitant with motile activities at the leading edge.

DISCUSSION

Fluorescence spectroscopy has long been a powerful approach for studying the binding and conformational changes of protein molecules. In addition, fluorescent analog cytochemistry allows direct observations of the behavior of specific molecules following microinjection into living cells [Wang, 1992; Taylor et al., 1992]. However, many proteins are not readily amenable to these approaches, due to problems in purification, lack of exposed reactive sites, or deactivation during the fluorescent labeling reaction. The use of site-directed mutagenesis and gene expression offers an alternate method for overcoming such limitations. Not only is it possible to obtain a large amount of proteins, but also to create reactive sites to enhance fluorescent conjugation. The new reactive site can be positioned either away from functional domains, to preserve biological activities, or next to sites of binding or post-translational modification, to create "optical biosensors" for monitoring changes in conformation or local environment [Post et al., 1994].

Despite the presence of three cysteine residues in the rat profilin sequence, the preparation of profilin analog has been difficult due to the low reactivity of this protein in its native form. Although a pyrene labeled profilin has been reported [Janmey, 1991], the conjugate was not fully characterized and the fluorescence characteristics of pyrene are not suitable for applications in living cells. In the present study, we have prepared fluorescent profilin by replacing the serine residue in rat profilin with a reactive cysteine. The creation of mutant profilin was facilitated by the feasibility of expressing functional rat profilin in *E. coli* [Babcock and Rubenstein, 1989], and by the detailed information on the primary, secondary, and tertiary structures of the molecule [Metzler et al., 1993; Cedergren-Zeppezauer et al., 1994; Schutt et al., 1993]. To create a functional fluorescent analog, the site of Serine 41 was chosen based on its distance from known ligand binding domains [Schutt et al., 1993; Vinson et al., 1993; Metzler et al., 1994]. In addition, the site shows diversity in evolution; the site

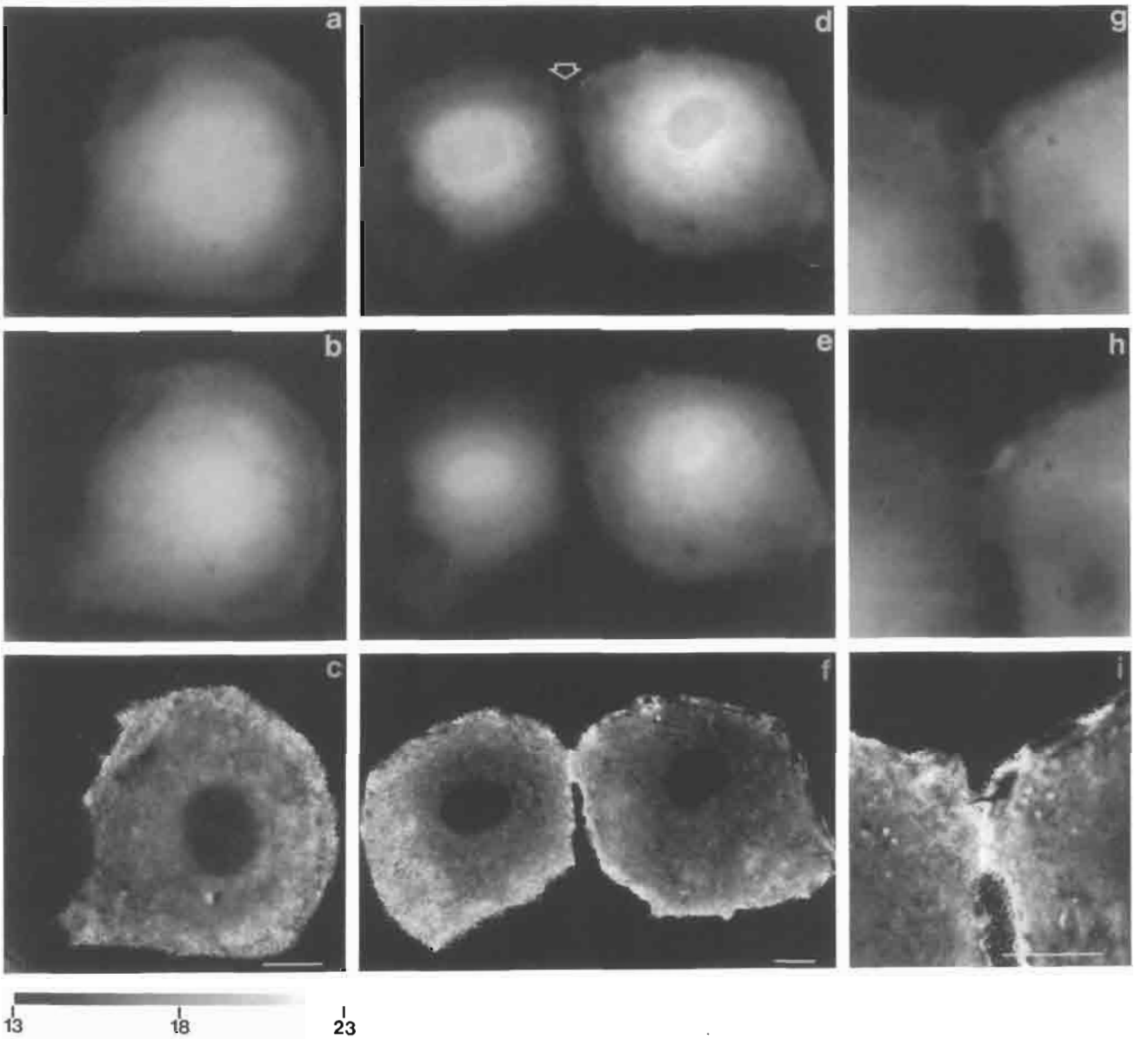

Fig. 6. Distribution of microinjected profilin relative to dextran. NRK cells were injected with a mixture of fluorescent profilin and dextran. Images of the low molecular weight dextran (**b, e, h**) indicate that it can readily enter the nucleus. Images of profilin (**a, d, g**) show a much more limited partition into the nucleus and a stronger signal in the anterior region. In addition, profilin is localized at cell-cell junction regions (d, arrow) (higher magnification, g, h, i). **c, f,** and **i** show ratio images of profilin against dextran. The ratio values (in arbitrary unit) corresponding to gray levels are indicated in the insert. Profilin appears to be enriched in the anterior region and depleted from the nucleus. Bar = 10 μm.

is occupied by an asparagine residue in bovine profilin [Edamatsu et al., 1991] and is relatively exposed to the solvent as indicated in the crystal structure [Cedergren-Zeppezauer et al., 1994].

Our binding assays indicate that labeled profilin has maintained its ability to interact with polyproline and actin. The K_d of actin-profilin binding for both wild type profilin and labeled profilin fell in the range of 3 to 5 μM, in agreement with values reported previously [Lal and Korn, 1985]. Binding of labeled profilin to actin or polyproline caused no significant change in the excitation and emission spectra, suggesting that there is no significant change in the environment surrounding the probe. Compared to the wild type profilin, the fluorescent profilin shows also a similar ability to inhibit the assembly of actin filaments and to stimulate the nucleotide exchange of actin subunits.

The binding assays further indicate that the fluo-

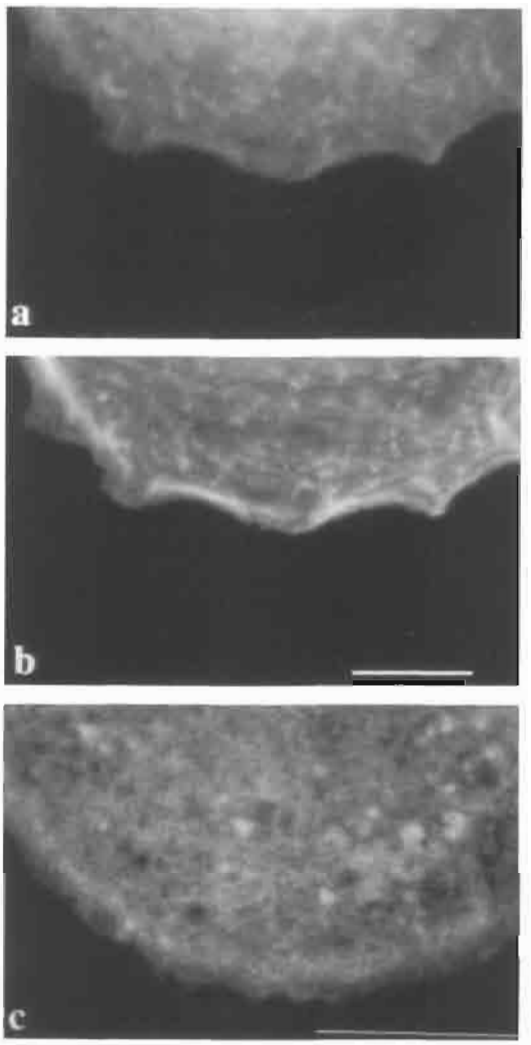

Fig. 7. Colocalization of injected profilin with vitamin D-binding protein staining. Cells were microinjected with labeled profilin, allowed to reach a steady state, and fixed and stained with fluorescein labeled vitamin D-binding protein. Profilin (**a, c**) is localized at part of the leading edge and at punctate structures behind the leading edge. Staining with vitamin D-binding protein (**b**) shows a very similar pattern of distribution and colocalization with profilin at most punctate structures. c shows a confocal image of a different cell focused near the cortex. In addition to punctate structures some faint network like structures can also be discerned. Bar = 10 μm

rescent profilin has maintained its ability to bind PIP_2. Surprisingly, the fluorescence of rhodamine was strongly quenched upon PIP_2 binding. A fluorophore of similar chemical structure, fluorescein, shows dramatic quenching upon binding to anti-fluorescein antibodies [Watt and Voss, 1977; Voss and Watt, 1977], likely due to the entry of the fluorophore into a highly hydrophobic environment. The quenching of fluorescence suggests that PIP_2 either interacts directly with the fluorophore or causes conformational changes near the Cysteine 41 site. The former is possible even if the site of labeling is not involved in PIP_2 binding [Raghunathan et al., 1992; Sohn et al., 1995], since PIP_2 is known to interact with profilin in an aggregated form [Lassing and Lindberg, 1985, 1988; Machesky and Pollard, 1993].

As compared to low molecular weight dextran, which serves as a marker for the accessible volume in the cell, the fluorescent profilin showed several significant features following microinjection into living cells. First, while low molecular weight dextrans entered freely into the nucleus and exhibited a high nuclear fluorescence intensity, fluorescent profilin showed a much lower intensity in the nucleus. This is likely due to the interactions of profilin with actin or other structures outside the nucleus, or to preferential interaction between profilin and PIP_2 in the nucleus. Second, injected profilin was incorporated into discrete punctate structures behind the lamellipodia, which were previously identified as foci of unpolymerized actin by labeling cells with fluorescent vitamin D-binding protein [Cao et al., 1993]. Thus profilin may play a role in sequestering actin subunits in these structures. Third, injected profilin showed a discrete region of high profilin fluorescence towards the anterior of the cell, likely due to a local concentration of profilin or decreased interactions with PIP_2 molecules. Our observations appear consistent with previous immunofluorescence observations indicating the concentration of profilin near the leading edge of cultured fibroblasts [Buß et al., 1992a,b]. In addition, profilin localization may explain why the distribution of different fluorescent analogs of actin differs according to their ability to interact with profilin: those capable of interacting with profilin localize more preferentially to the anterior region of the cell [Giuliano and Taylor, 1994]. Together, these results suggest that profilin may be involved in signaling or regulating the protrusion of the leading edge [Aderem, 1992; Sohn and Goldschmidt-Clermont, 1994], and that cell polarity may be established through a biased localization of profilin in the anterior region.

Interestingly, profilin did not appear to concentrate as a continuous, bright band along the leading edge of the cell, as one might expect if it is required for maintaining the active assembly of actin filaments in this region. However, such activity could be achieved without a conspicuous, stable concentration of profilin [Reinhard et al., 1995]. We have on the other hand detected a continuous band of injected profilin at sites of cell-cell adhesion. While the functional role of profilin in cell adhesion is unclear, recent studies indicated that profilin can bind to proteins with proline-rich domains at adhesion sites [Reinhard et al., 1995].

Besides known interactions with actin and several ligands, recent studies indicated that profilin can interact with a whole set of proteins including non-conventional

actin [Machesky et al., 1994]. The present fluorescent analog should serve as a useful probe for testing the interaction of profilin with various proteins or cellular structures in vitro and in living cells. Of particular importance will be the establishment of the physiological significance of profilin-polyproline interactions and profilin-PIP_2 binding. For such studies one may take advantage of the sensitivity of the fluorescence of the present probe to PIP_2 binding, or generate additional analogs with fluorophores located at sites sensitive to other binding interactions. Furthermore, fluorescent analogs of mutant profilins that are defective in various binding domains may be generated to determine how such mutations affect its overexpression phenotype, its distribution in living cells, and its response to signaling mechanisms.

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