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Fluorescence Resonance Energy Transfer (FRET)-based Detection of Profilin–VASP Interaction

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Abstract

Profilins belong to a family of small G-actin binding proteins which are thought to assist in F-actin elongation at the leading edge of migrating cells through their interactions with a host of actin-binding proteins including Ena (enabled)/VASP (vasodilator stimulated phosphoprotein). Profilin's interactions with the major actin regulators have been studied almost exclusively using biochemical methods. Therefore spatiotemporal features of these protein–protein interactions have not been resolved so far. In this paper, we for the first time demonstrate the feasibility of GFP-based fluorescence resonance energy transfer (FRET) technique to detect VASP's interaction with profilin-1, a ubiquitously expressed member of profilin family of genes. Specifically, we performed acceptor photobleaching FRET in MDA-MB-231 breast cancer cells to show prominent VASP–Pfn1 interaction at the membrane ruffles near the leading edge.

Keywords

VASP; Profilin; FRET; Acceptor photobleaching; MDA-MB-231 cells; Breast cancer

Introduction

Cell migration is a multistep event, which proceeds in a cyclic manner consisting of discrete events e.g. cell protrusion, stabilization of protrusion by formation of cell-substrate adhesion, and generation of actomyosin-based traction force (contractility) to propel cell body forward with subsequent release of the rear of the cell.¹⁹ Lamellipodial protrusion, the defining step of cell migration, is propelled by actin polymerization involving de novo actin nucleation followed by filament elongation and/or elongation of pre-existing filaments. Proteins belonging to Neuronal Wiskott Aldrich Syndrome Protein (N-WASP), Ena (Enabled)/VASP (vasodilator stimulated phosphoprotein) and Formin families are considered to be major molecular players for actin nucleation and/or filament elongation at the leading edge resulting in formation of actin-based protrusive structures such as lamellipodia and filopodia.¹⁴ A common feature of all of these proteins is the presence of proline-rich domains which are able to recruit profilin(Pfn)–actin complex (Pfns—a family of small G-actin binding proteins that also display affinity for polyproline sequences).²¹

Mammalian genome contains four Pfn1 genes of which Pfn1 is the founding member and the ubiquitously expressed form while the other variants of Pfns, namely Pfn2, Pfn3, and Pfn4, are expressed in a tissue-restricted manner.⁸ Depletion of Pfn1 results in reduced overall F-actin level in several different cell types thus indicating that Pfn1 is an essential regulator of actin polymerization *in vivo*.^{4,5,23} Fluorescence localization of either endogenous Pfn1^{3,11} or ectopically expressed GFP-Pfn in cultured cells²² has revealed punctuate distribution of Pfn throughout cytoplasm and nucleus with some degree of accumulation at the cell periphery. Pfn1 depletion reduces the speed of membrane protrusion and intracellular propulsion velocity of infecting pathogens inside host cells/cell extracts (a molecular mimicry of actin-based protrusion at the leading edge).^{1,4,5,12,13} Together these findings demonstrate a key function of Pfn1's role in membrane protrusion during cell migration.

Our recent study has shown that both actin and polyproline interaction of Pfn1 are important for membrane protrusion and overall cell migration.⁵ N-WASP-dependent generation of actin microspikes and intracellular motility of bacterial pathogens require their polyproline domains and functional interactions with Pfn1.^{13,20} Ena/VASP-Pfn1 interaction is required for efficient motility of bacterial pathogens and neurite outgrowth.^{7,10} Therefore, Pfn's interactions with Ena/VASP and N-WASP proteins are likely to play critical role in actin assembly at the leading edge. VASP has a stronger preference for binding to Pfn1-actin complex than Pfn1 alone.⁶ Therefore, proline-rich actin regulators such as N-WASP and Ena/VASP are thought to act as molecular scaffolds to recruit Pfn1-actin complex to facilitate actin nucleation and elongation at the protruding membrane during cell migration. Although important, these protein-protein interactions have been mostly confirmed by biochemical methods and never been directly visualized in cells. In this study, we establish for the first time the feasibility of imaging VASP-Pfn1 interaction by GFP-based acceptor photobleaching fluorescence resonance energy transfer (FRET) technique.

Methods

Plasmid Construction

Generation of GFP-Pfn1 has been previously described.¹⁷ Pfn1 was excised from EGFP-Pfn1 vector and subcloned into EYFP-C1 vector at *Hind3* and *BamH1* restriction sites to generate YFP-Pfn1 construct. YFP-Pfn1 was further subcloned into ECFP-N1 vector at *EcoR1* and *Kpn1* restriction sites to generate the chimeric YFP-Pfn1-CFP (YPC) construct. A myc-tagged YFP-Pfn1 construct (myc-YFP-Pfn1) was generated by subcloning YFP-Pfn1 into a pCMV-myc expression vector at *EcoR1* and *Kpn1* restriction sites. H133S substitution in Pfn1-coding region of YFP-Pfn1 and myc-YFP-Pfn1 was performed by site-directed mutagenesis as previously described.²³ Full-length VASP cDNA (PCR amplified from a human cDNA library) was subcloned into ECFP-C1 vector at *Hind3* and *BamH1* restriction sites. Pfn1-shRNA was constructed by ligating a commercially available vector pRFP-C-RS (Origene Technologies Inc., Rockville, MD) with a custom-designed 21-mer hairpin sequence targeting Pfn1 at *BamH1* and *Hind3* sites. The sense sequence for the Pfn1-shRNA is 5'-GAT CGA AGA AGG TGT CCA CGG TGG TTA AGT TCT CTA ACC ACC GTG GAC ACC TTC TTT TTT TTG GAA A-3'; the antisense sequence is 5'-AGC TTT TCC AAA AAA AAG AAG GTG TCC ACG GTG GTT AGA GAA CTT AAC CAC CGT GGA CAC CTT CTT C 3'. A control shRNA vector against luciferase (luc-shRNA) was also constructed in the same manner with the sense sequence: 5'-GAT CGG CTT ACG CTG AGT ACT TCG ATT CAA GAG ATC GAA GTA CTC AGC GTA AGC TTT TTT GGA AA-3', and the antisense sequence: 5'-AGC TTT TCC AAA AAA GCT TAC GCT GAG TAC TTC GAT CTC TTG AAT CGA AGT ACT CAG CGT AAG CC-3'. All constructs were confirmed by DNA sequencing.

Cell Culture and Transfection

HEK293 cells were cultured in DMEM-F12 media supplemented with 10% fetal bovine serum (FBS) and antibiotics. MDA-MB-231 (MDA-231) breast cancer cells, stably transfected with Pfn1- and Luc sh-RNA, were selected and maintained in EMEM supplemented with 10% FBS, Na-pyruvate, antibiotics and 250 ng/mL puromycin. Plasmid transfection of cells was performed using lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Immunoprecipitation/Immunoblotting

Total cell lysate was prepared by extracting cells with modified RIPA buffer (50 mM Tris-HCl—pH 7.5, 150 mM NaCl, 1% triton-X100, 0.25% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 50 mM NaF, 1 mM sodium pervanadate, and protease inhibitors). For immunoprecipitation, 1000 μ g of lysate (extracted using a similar lysis buffer without SDS) was incubated overnight with 4 μ g of polyclonal GFP antibody (Abcam, Cambridge, MA) and then with 25 μ L of protein-G/protein A-conjugated agarose beads for an additional hour. Immunoprecipitated protein sample was washed 3 times with the lysis buffer, resuspended in 25 μ L of 2 $\times$ sample buffer and run on a SDS-PAGE. Immunoblotting conditions for the various antibodies were: monoclonal GFP (Pharmingen, San Diego, CA—1:1000), monoclonal VASP (Pharmingen, San Diego, CA—1:1000), polyclonal Pfn1 (Santa Cruz Biotechnology, Santa Cruz, CA—1:1000) and monoclonal GAPDH (Abd Serotec, Raleigh, NC—1:2000).

FRET

For spectral FRET analyses, cells expressing various FRET constructs were trypsinized and resuspended in PBS before performing an emission scan of cell suspension for a wavelength range of 465–600 nm corresponding to excitation at 425 nm using a spectrofluorophotometer (spectral scan was obtained within 10 min of trypsinization). In imaging experiments, cells expressing FRET constructs were allowed to form a monolayer which was then scratched using a pipet-tip. Cells were allowed to migrate for 6–8 h before fixing with 4% formaldehyde. Acceptor photobleaching was then performed on cells at the edge of the wound (these cells display polarized morphology and tend to be most motile before fixation) on a Zeiss LSM 510 Meta confocal microscope (Carl Zeiss, Inc., Thornwood, NY) coupled to an argon laser. Cells were bleached on the region of interest (ROI) at 514 nm wavelength with 50% laser power, and the bleaching duration ranged from 10 to 20 s (for partial bleaching) to ~1 min (for whole cell bleaching). Cells were imaged with a 63 $\times$ 1.4 NA Zeiss oil immersion objective at either 2 $\times$ or 4 $\times$ zoom. CFP and YFP images were acquired with laser power set at 33% and 7.5%, respectively. To calculate FRET efficiency, multiple 120-pixel long lines within ROI were constructed from the edge of the cell into the cytoplasm; the average intensity of these line scans in CFP channel before bleaching was subtracted from the corresponding value recorded after bleaching and then normalized to the pre-bleaching value.

Results and Discussion

Pfn1's association with Ena/VASP proteins has been previously examined biochemically.¹⁵ However, biochemical techniques do not allow spatial visualization of protein–protein interactions in cells. The overall objective of this study was to determine whether FRET can be used to visualize VASP–Pfn1 interaction in cells. FRET involves energy transfer from an excited fluorescent molecule, the donor, to a second proximal and unexcited fluorescent molecule, the acceptor, such that the acceptor fluoresces and the emission of the donor diminishes. When two potentially interacting proteins are conjugated to the appropriate

donor and acceptor fluorophores, their direct association can be detected by FRET.¹⁸ Figure 1a schematically represents the various constructs that we generated for a CFP (donor)–YFP (acceptor)-based FRET system. Specifically, we created N-terminus fusion of CFP and YFP to VASP and Pfn1, respectively as the potential FRET pairs. Even though fusing GFP to the N-terminus (analogous to our case) causes a ~50% reduction in polyproline binding with no significant effect on actin and phospholipid binding of Pfn1, it is still a reasonable strategy for FRET experiments since the alternative C-terminal fusion of GFP completely abolishes Pfn1's polyproline binding. Also GFP-Pfn1 localizes to subcellular regions with high actin dynamics similar to endogenous Pfn1, while Pfn1-GFP is severely deficient in this function.²² Fusion of GFP to the N-terminus of VASP has been previously adopted for a live-cell reporter for VASP.¹⁶ As a negative control for FRET experiment, we created H133S point mutation in the coding region of Pfn1 on YFP-Pfn1. This particular point mutation severely impairs polyproline interaction of Pfn1²³ and therefore this mutated form of Pfn1 should have very low affinity, if at all, for VASP binding. For biochemical experiments, we further modified YFP-Pfn1 or its H133S mutant to include a myc-tag at their N-termini. Since anti-GFP antibody indiscriminately recognizes both CFP and YFP, addition of a myc-tag enables specific immunoprecipitation of YFP-Pfn1 by anti-myc antibody and detection of its interaction with CFP-VASP. This step was necessary to biochemically confirm that adding CFP-tag does not impair VASP's ability to bind to YFP-Pfn1. As a positive control for FRET, we generated a chimeric YFP–Pfn1–CFP construct (YPC – CFP and YFP being present on the same construct, should be able to produce strong intramolecular FRET). Figure 1b shows expression of various constructs following transient transfection in HEK293 cells.

To confirm *in vivo* binding between CFP-VASP and myc-YFP-Pfn1, we co-expressed these constructs in HEK293 cells by transient transfection (in negative control experiments, CFP-VASP was co-expressed with myc-YFP-Pfn1-H133S or CFP was co-expressed with myc-YFP-Pfn1). When anti-myc immunoprecipitates from these two transfection groups were probed with GFP antibody, CFP-VASP co-precipitated with only myc-YFP-Pfn1. This is specifically due to interaction between VASP and Pfn1 since (i) CFP-VASP failed to coprecipitate with polyproline-binding deficient myc-YFP-Pfn1-H133S as expected, and (ii) CFP did not co-precipitate with myc-YFP-Pfn1 (rules out formation of possible CFP–YFP heterodimers) (Fig. 2). These results confirm that adding CFP or YFP tags still preserve the *in vivo* interaction between VASP and Pfn1.

Next, to determine whether VASP–Pfn1 interaction can be detected by spectral FRET, we co-expressed CFP-VASP with either myc-YFP-Pfn1 or myc-YFP-Pfn1-H133S (a negative control for FRET interaction) in HEK293 cells. As additional negative controls, either CFP-VASP was expressed alone or CFP was co-expressed with YFP-Pfn1. As a positive control, YPC was expressed alone in HEK293 cells. We then excited cell suspension at 425 nm wavelength (excitation maxima for CFP) and obtained the resultant emission spectrum over wavelengths ranging from 460 to 600 nm at 5 nm intervals. We reasoned that if there is a transfer of energy from CFP to YFP due to protein–protein interaction, we should see two emission peaks, one at ~465–470 nm (corresponds to the emission maxima for CFP) and the second one at ~520–525 nm (corresponds to the emission maxima for YFP). Figure 3a shows the emission spectra data from the different transfection groups. Consistent with the spectral characteristic of CFP, cells expressing CFP-VASP alone showed only a single peak at 470 nm. Cells co-expressing CFP-VASP with myc-YFP-Pfn1 showed the FRET signature of emission peak at both 465 and 520 nm wavelength (this signature was also seen in our positive control YPC expressers). Detection of the second peak at 525 nm was specifically due to FRET interaction between VASP and Pfn1 since two important negative control groups of cells (CFP-VASP/myc-YFP-Pfn1-H133S and CFP/myc-YFP-Pfn1 coexpressers) did not display that energy transfer signature. We performed these spectral FRET

experiments with cells in suspension for technical reasons. However, since suspended cells do not mimic the natural condition for non-hematopoietic cells such as HEK293 cells, one should not rule out that spectral FRET signal between CFP-VASP and YFP-Pfn1 is due to possible non-physiological increase in VASP–Pfn1 interaction when cells are switched from adherent to suspension states. To examine whether cell adhesion status (i.e. suspension vs. adherent state) has any influence on VASP–Pfn1 interaction in cells, we expressed GFP-Pfn1 in HEK293 cells by transient transfection and examined endogenous VASP binding to immunoprecipitated GFP-Pfn1 from either adherent or suspended cells. Figure 3b shows that binding of VASP to GFP-Pfn1 actually decreases dramatically upon loss of cell-substrate adhesion. Based on this observation, we presume that our detected spectral FRET signal between CFP-VASP and YFP-Pfn1, if at all, is an underestimate of the actual FRET between the two proteins in a more physiologically relevant “adherent” state of cells. These data validate that *in vivo* VASP–Pfn1 interaction can be detected by spectral FRET.

We next asked whether FRET interaction between VASP and Pfn1 can be visualized in cells. As a cell line of choice, we used MDA-MB-231 (MDA-231) breast cancer cells since these cells are highly motile in nature and display a polarized (i.e. front to back asymmetry) morphology in culture. We also reasoned that endogenous Pfn1 should compete with exogenous YFP-Pfn1 for binding to CFP-VASP and lower the FRET signal which may increase the common difficulty of detecting the interaction by imaging technique. Therefore, we chose to perform FRET imaging experiments in a suppressed endogenous Pfn1 background. We generated clones of MDA-231 cells stably expressing either Pfn1-shRNA or luc-shRNA (control). Immunoblot in Fig. 4a shows strong suppression of Pfn1 expression in Pfn1-shRNA expressers. We also introduced silent mutation in the Pfn1-shRNA targeting region of YFP-Pfn1 as previously described⁵ so that this acceptor construct can be expressed in the continued presence of Pfn1-shRNA. Also, since MDA-231 cells do not express detectable level of Pfn2.²³ Therefore functional competition from Pfn2 is not a concern. On the other hand, mammalian cells express three members of Ena/VASP proteins (Mena, VASP and Evl) which have similarities in cellular localization and shared ligands. It is therefore technically difficult to minimize competitions from other members of Ena/VASP proteins (we have unpublished observation that expressing GFP-tagged VASP in MDA-231 cells does not affect endogenous VASP expression). Even though the most widely adopted FRET imaging technique involves detection of acceptor emission following donor excitation, bleed-through of donor emission into acceptor channel and cross-excitation of acceptor molecular by donor-specific excitation wavelength require mathematical correction of raw FRET images to extract the real FRET signal. A problem with this approach is that FRET signal, if low, can be masked by donor bleed-through and acceptor cross-excitation components (this is an issue particularly for CFP/YFP-based system because of large spectral overlaps). We here chose acceptor photobleaching FRET technique which relies on the principle that energy transfer between donor and acceptor is reduced when the acceptor (YFP in this case) is photobleached resulting in an increase in donor (CFP in this case) fluorescence. This technique does not require any rigorous mathematical image correction as needed for the conventional FRET imaging and is also a better diagnostic for protein–protein interaction since fluorescence, in general, decreases due to photobleaching unless there is a FRET.⁹ Figure 4b shows a set of representative pre- and post-bleach CFP- and YFP-channel images of Pfn1-shRNA cells expressing CFP-VASP with either YFP-Pfn1 or YFP-Pfn1-H133S (the region outlined by the box shows a dramatic decrease in YFP fluorescence after cells were subjected to photobleaching pulses thus confirming the bleaching of YFP fluorophore). When we compared CFP fluorescence of the ROI between pre- and post-bleach conditions in CFP-VASP/YFP-Pfn1 coexpressers, we saw an increase in fluorescence intensity (as apparent from the pseudocolor plot of the ROI) thus indicating FRET between VASP and Pfn1. The corresponding change in CFP fluorescence in CFP-VASP/YFP-Pfn1-H133S coexpresser, was very minimal thus indicating low or no FRET as

expected. This was quantitatively validated by significant difference in the average FRET efficiency between the two experimental conditions (20% for CFP-VASP/YFP-Pfn1 co-expressers vs. -0.8% for CFP-VASP/YFP-Pfn1-H133S co-expressers). Note that a negative number means that post-bleach value is lower than the pre-bleach value and this is not unusual particularly in a negative-control FRET condition since some degree of photobleaching can occur as a result of successive imaging of a fluorophore.⁹

Next, to determine the subcellular location of VASP-Pfn1 interaction in cells, we chose CFP-VASP/YFP-Pfn1 co-expressers and bleached the entire cell at YFP-specific wavelength. Pseudocolor plots of CFP channel images show increase in CFP-fluorescence after photobleaching at the membrane ruffles in the protruding lamellipod (arrows) and to some extent in a diffused-fashion in perinuclear region (arrowheads). Overall decrease in YFP-fluorescence after photobleaching confirms partial bleaching of YFP fluorophore as desired (Fig. 5—upper panel). To further confirm that the increase in CFP-fluorescence is due to FRET and not caused by a random drift in fluorescence (possibly due to change of focus during bleaching), we chose similar co-expressing cells and acquired two successive sets of CFP and YFP images except those cells were not actually photobleached (note that the time interval between the two successive CFP or YFP images was similar for photobleached and non-bleached cells). In the absence of photobleaching, pseudocolor plots of CFP and YFP images did not show any detectable change between the two sets of images (Fig. 5—lower panel). These data further establish the spatial specificity of our FRET images. Membrane ruffles near the leading edge represent regions of high actin dynamics. In fibroblasts, it was demonstrated that Ena/VASP activity leads to faster membrane protrusion, but Ena/VASP-directed protrusions tend to be unstable and are subjected to more frequent retractions.² Since unproductive protrusions often lead to membrane ruffles, localization of VASP-Pfn1 interaction in those regions is not unexpected. The significance of VASP-Pfn1 FRET in a broad perinuclear region is not clear at this point.

In summary, this study is the first successful demonstration of the use of an intermolecular FRET to detect and spatially resolve interaction of Pfn1 with one of its major binding partners. Although acceptor photobleaching is a useful FRET technique to spatially localize protein-protein interaction in cells, it is not suitable for studying dynamics of interaction. Future studies should focus on studying spatiotemporal dynamics of VASP-Pfn1 interaction during cell migration using a more sophisticated and accurate fluorescence lifetime imaging measurements (FLIM)-based FRET technique.

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FIGURE 1.
 (a) Schematics of various FRET constructs. (b) GFP-immunoblot shows expression of various FRET constructs 48 h following transient transfection in HEK293 cells.

FIGURE 2.

Myc-YFP-Pfn1 or its H133S mutant (coexpressed with either CFP or CFP-VASP in HEK293 cells), when immunoprecipitated with anti-myc antibody and probed with GFP-antibody, shows binding between CFP-VASP and myc-YFP-Pfn1. No binding was detected either between CFP and myc-YFP-Pfn1 or between CFP-VASP and myc-YFP-Pfn1-H133S (the arrow shows the heavy chain IgG band of the antibody used for immunoprecipitation) (TCL—total cell lysate; IP: immunoprecipitation).

FIGURE 3.

(a) Emission spectra of cells expressing various FRET constructs (excitation: 425 nm). FRET peak is identified by the arrow. Cells expressing YPC (positive control) or coexpressing CFP-VASP with myc-YFP-Pfn1 only show evidence of spectral FRET (all intensity values are normalized with respect to the peak value at around 465–470 nm). (b) VASP blot (top panel) shows a dramatic difference in binding of VASP to GFP-Pfn1 IPs between suspension (susp) and adherent (adh) states of HEK-293 cells. GFP blot in the lower panel serves as a loading control for TCL and IP samples (TCL—total cell lysate; IP: immunoprecipitation).

FIGURE 4.

(a) Pfn1 immunoblot of TCL derived from parental (WT) and various stable shRNA-expressers of MDA-231 cells show strong suppression of Pfn1 expression in Pfn1-shRNA expressers (luc—luciferase). GAPDH blot serves as the loading control. (b) Pre- and post-bleach images of two sets of MDA-231 co-expressers (CFP-VASP/YFP-Pfn1 and CFP-VASP/YFP-Pfn1-H133S) in CFP and YFP channels (boxes outline the region of bleaching and insets show pseudocolor plots of the bleached region). (c) A box and whisker plot comparing the average FRET efficiency between the two experimental conditions. In this plot asterisk represents the mean, middle lines of box indicates median, top of the box indicates 75th percentile, bottom of the box measures 25th percentile and the two whiskers indicate the 10th and 90th percentiles, respectively (n—number of analyzed cells pooled from 2 to 3 experiments; ex: excitation, em: emission; bar—20 μ m).

FIGURE 5.

Pseudocolor plots of CFP and YFP images of a cell co-expressing CFP-VASP and YFP-Pfn1 before and after photobleaching of the whole cell (Top panel—representative image of 10 cells). Arrows indicate FRET at membrane ruffles. Arrowhead shows some extent of FRET in a diffused fashion in perinuclear region. The bottom panel shows similar sets of images acquired for a cell which is not actually photobleached (representative image of 3 cells) (ex: excitation, em: emission; bar—20 μ m).