A new method for direct detection of the sites of actin polymerization in intact cells and its application to differentiated vascular smooth muscle

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Kim HR, Leavis PC, Graceffa P, Gallant C, Morgan KG. A new method for direct detection of the sites of actin polymerization in intact cells and its application to differentiated vascular smooth muscle. Am J Physiol Cell Physiol 299: C988–C993, 2010. First published August 4, 2010; doi:10.1152/ajpcell.00210.2010.—Here we report and validate a new method, suitable broadly, for use in differentiated cells and tissues, for the direct visualization of actin polymerization under physiological conditions. We have designed and tested different versions of fluorescently labeled actin, reversibly attached to the protein transduction tag TAT, and have introduced this novel reagent into intact differentiated vascular smooth muscle cells (dVSMCs). A thiol-reactive version of the TAT peptide was synthesized by adding the amino acids glycine and cysteine to its N-terminus and forming a thionitrobenzoate adduct: viz. TAT-Cys-S-STNB. This peptide reacts readily with G-actin, and the complex is rapidly taken up by freshly enzymatically isolated dVSMCs, as indicated by the fluorescence of a FITC tag on the TAT peptide. By comparing different versions of the construct, we determined that the optimal construct for biological applications is a nonfluorescently labeled TAT peptide conjugated to rhodamine-labeled actin. When TAT-Cys-S-STNB-tagged rhodamine actin (TSSAR) was added to live, freshly enzymatically isolated cells, we observed punctae of incorporated actin at the cortex of the cell. The punctae are indistinguishable from those we have previously reported to occur in the same cell type when rhodamine G-actin is added to permeabilized cells. Thus this new method allows the delivery of labeled G-actin into intact cells without disrupting the native state and will allow its further use to study the effect of physiological intracellular Ca2+ concentration transients and signal transduction on actin dynamics in intact cells.

MATERIALS AND METHODS

Peptide synthesis. A modified HIV-TAT protein transduction domain comprising residues YGRKKRRQRRR was synthesized in the solid state using an Applied Biosystems model 433A peptide synthesizer. Fluorenylmethoxycarbonyl was employed to block α-amino groups. Coupling of protected amino acids to the nascent peptide was accomplished by converting their α-carboxyl groups to active benzotriazole esters using the coupling reagents N-hydroxybenzotriazole/2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HOBt/HBTU).

To generate a thiol-reactive version of TAT, we added the amino acids glycine and cysteine to the N-terminus of the TAT sequence. The HPLC-purified peptide (Fig. 1A,a) was conjugated to 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) (Fig. 1A,b) to yield the mixed disulfide TAT-Cys-S-STNB (Fig. 1A,c) plus the thionitrobenzoate anion (Fig. 1A,d), which is strongly colored and can be quantified from its absorption of light at 412 nm. The mixed disulfide was purified by reversed-phase HPLC and reacted readily with protein (Fig. 1A,e) or peptide thios to yield disulfide-linked TAT conjugates whose formation can be followed by the release of the colored thionitrobenzoate anion from the peptide.

In some cases we added a fluorescent label to the peptide by constructing an orthogonal bridge consisting either of the sequence GGG-β-A-FITC or of ε-aminohexanoic acid-FITC on the side chain of the first lysine residue (K4) of the TAT sequence. The α-amino group of this residue was protected during the initial synthesis by the acid labile 4-methyltrityl group, which was subsequently removed from the peptide by five consecutive 1-min treatments with 2% trifluoroacetic acid plus 3% triisopropylsilane, leaving the peptide...
still attached to the resin with all other reactive side chains still protected. The FITC was coupled to the amino group of the β-Ala or the ε-amino group of the aminohexanoic acid. The peptides were cleaved and purified to >95% using reversed-phase HPLC.

Actin preparation and labeling. Actin and rhodamine-labeled actin were prepared as previously described (11). Before being reacted with the TAT-Cys-S-STNB or FITC-TAT-Cys-S-STNB peptide, rhodamine-actin was treated with 5 mM dithiothreitol (DTT) for 30 min and dialyzed exhaustively versus G buffer (2 mM MOPS, 0.2 M CaCl₂, 0.2 mM ATP, 0.01% NaN₃, pH 7.5). This step assures that actin Cys374 is fully reduced and able to react with the peptides by disulfide exchange. The reduced rhodamine-actin was labeled with the TAT-STNB or FITC-TAT-STNB peptide by reacting at a peptide-to-actin molar ratio of 2.5 for overnight at 4°C in G buffer plus 0.3 M KCl. The labeled actin was then dialyzed exhaustively against G buffer. This doubly labeled actin was often cloudy and was cleared by centrifugation at about 90,000 g. The FITC-to-actin molar ratio of the product FITC-TAT-S-S-actin-rhodamine (FTSSA) was determined from the peak absorbance at 500 nm using an extinction coefficient of $5 \times 10^4$ M$^{-1}$, after correcting for the overlapping rhodamine absorbance. The labeling ratio varied between 0.65 and 1.0. The rhodamine labeling ratio of FTSSAR was similarly determined from the peak absorbance at about 600 nm using an extinction coefficient of $5 \times 10^4$ M$^{-1}$, after correcting for the overlapping FITC absorbance. The labeling ratio varied between 0.4 and 0.9. The TAT labeling ratio for the rhodamine-actin + NbSS-TAT product, i.e., TSSAR, was not readily determined.

Light scattering experiments. Actin polymerization was followed by measuring the accompanying increase in light scattering (6, 8). Light scattering was measured as a function of time in a Varian Eclipse spectrofluorometer using an excitation and emission wavelength of 350 nm. Polymerization was initiated by adding a small aliquot of a concentrated NaCl/MgCl₂ solution to 5 mM actin in G buffer to bring the solution to 40 mM NaCl/2 mM MgCl₂ (resulting in the formation of F actin buffer). In some cases DTT was present at 10 mM. Temperature was controlled at 20°C during the experiments.

Cell preparation and imaging. All procedures in this study were performed according to protocols approved by the Institutional Care Fig. 1. Synthesis and delivery of FITC-TAT labeled actin into differentiated vascular smooth muscle (dVSM) cell. A: synthesis of TAT-tagged, labeled actin (see text for details). B: freshly isolated aorta cell loaded with fluorescein-TAT-actin demonstrates intracellular localization of FITC-TAT-S-S-actin-rhodamine (FTSSA); calibration bar, 5 μm.
and Use Committee. Cells were freshly enzymatically dissociated from aorta tissue by a previously published method (10, 11). Briefly, ferrets (Marshall Farms, North Rose, NY) were euthanized by an overdose of isoflurane, and the aorta was quickly removed from animals and placed in an oxygenated physiological salt solution (PSS) consisting (in mM) of 120 NaCl, 5.9 KCl, 1.2 NaH₂PO₄, 25 NaHCO₃, 11.5 dextrose, 1.0 CaCl₂, and 1.4 MgCl₂ (pH = 7.4), and dissected under a dissecting microscope in oxygenated (95% O₂-5% CO₂) PSS to remove connective tissue and the endothelium. Then aorta tissue was cut into small pieces and placed into 7.5 ml Ca²⁺/Mg²⁺-free Hanks balanced salt solution (HBSS), 100 U/ml type II collagenase (Worthington), 1.0 U/ml elastase (Roche diagnostics), and 0.2% BSA (Sigma). The tissue pieces were filtered on a nylon mesh (pore size ~0.5 mm) and washed with 12 ml Ca, Mg-free HBSS, and 0.2% BSA. The wash buffer containing the dissociated cells was poured over clean glass coverslips on ice under an atmosphere of 100% oxygen. One coverslip of each batch was used to confirm that the cells were capable of shortening. All remaining coverslips were put in a PSS containing 300 mM sucrose, which, as previously detailed (10), increases the tonicity of the solution and prevents stimulus-induced shortening directly at the crossbridge level allowing the use of the cells for imaging. Before the addition of labeled actin solution to the cells, coverslips were briefly washed three times by dipping in sucrose-Hanks buffer with 0.2% BSA to remove unattached cells. Labeled actin solution (FTSSAR or TSSAR, 10 μM) was added to coverslips for 5 or 20 min and then the cells were stimulated with 10 μM of phenylephrine for 5 min at room temperature under an atmosphere of 100% oxygen. One coverslip of each batch was used to confirm that the cells were capable of shortening. All remaining coverslips were put in a PSS containing 300 mM sucrose, which, as previously detailed (10), increases the tonicity of the solution and prevents stimulus-induced shortening directly at the crossbridge level allowing the use of the cells for imaging. Before the addition of labeled actin solution to the cells, coverslips were briefly washed three times by dipping in sucrose-Hanks buffer with 0.2% BSA to remove unattached cells. Labeled actin solution (FTSSAR or TSSAR, 10 μM) was added to coverslips for 5 or 20 min and then the cells were stimulated with 10 μM of phenylephrine for 5 min at room temperature under an atmosphere of 100% oxygen. After washing was completed, cells were fixed for 10 min with 0.5% glutaraldehyde with 0.2% Triton-X 100 in cytoskeletal buffer (50 mM NaCl, 30 mM sucrose, 10 mM PIPES, 3 mM MgCl₂, pH 6.8). Cells were then rinsed three times using TBS/T (0.15 M NaCl, 0.02 M Tris·HCl, 0.1% Triton-X 100, pH 7.4) and mounted with FluorSave (Calbiochem). In some cases, cells were counterstained with phalloidin (1:100 dilution, Alexa Fluor 350).

Images were recorded by a high-resolution fluorescence CCD camera (CoolSNAP HQ², Photometrics) with NIS-Elements Advanced Research (Nikon) software. For deconvolution microscopy, three-dimensional image stacks were acquired with a Nikon Eclipse TE 2000-E inverted microscope equipped with a Nikon Plan Apochromat ×60A (numerical aperture 1.4) oil immersion objective. Out-of-focus fluorescent blur in recorded images was removed by deconvolution of Z-stacks (Richardson-Lucy algorithm, constrained iterative-maximum likelihood estimation algorithm). In addition, for all costaining experiments it was confirmed that there was no detectable crosstalk between fluorescent labels by exchanging excitation/emission filters on single labeled coverslips.

RESULTS

Transduction peptides possess the inherent ability of being able to penetrate cell membranes themselves (9, 12), and, when attached to other peptides or proteins, render them membrane permeant as well. The three most commonly used transduction vehicles are the antennapedia peptide (4), derived from a family of Drosophila homeoproteins, the herpes simplex virus VP22 protein (28), and the HIV TAT protein transduction domain (25–27). Any of these sequences can be incorporated into a protein/peptide sequence either via recombinant techniques or by solid-state peptide synthesis. An alternative, and in some cases, more convenient, approach is to incorporate a...
reactive moiety, such as TNB into the TAT sequence so that it can be conjugated to a thiol group in a protein or peptide (Fig. 1A). An additional advantage would be to have a reversibly attachable TAT probe that should, in theory, dissociate from the cargo protein after entering the reducing environment of the cell. Thus our goal was, first, to synthesize such a construct with G-actin as the cargo. FITC-TAT-Cys-S-STNB peptide reacted readily with G-actin, and the complex was rapidly taken up by freshly enzymatically isolated dVSMC, as indicated by the green fluorescence of loaded cells (Fig. 1B) compared with undetectable fluorescence of unloaded cells.

We then reacted the actin with rhodamine and subsequently labeled the rhodamine-actin conjugate with the FITC-TAT-Cys-S-STNB with the expectation that the peptide would dissociate from rhodamine-labeled actin and disperse once within the cell, whereas the actin would incorporate into punctae or filamentous structures and that this would be visualized as a lack of colocalization of the FITC and rhodamine probes. However, the FITC probe exhibited an intracellular distribution quite similar to that of the rhodamine-actin (Fig. 2). This result suggested that the intracellular environment may not have reduced the disulfide linkage connecting the peptide to the actin. However, even when DTT (10 mM for 10 min) was added to the cells to ensure reduction of the disulfide, the apparent colocalization of the two fluorophores persisted (Fig. 3). These results, taken together, raised the possibility that the FITC-labeled TAT may remain nonspecifically associated with the actin even after the disulfide linkage between the two is disrupted. This possibility was confirmed by passing actin that had been labeled with and subsequently reduced by the addition of DTT through a gel filtration column (TSK G3000 SWx1, Tosoh, Japan). The UV absorbance of the effluent was monitored both at 280 and 490 nm, the absorption maxima of actin and fluorescein, respectively. Actin and fluorescein coeluted from the column (data not shown) indicating that the latter remained associated with the actin.

An important question for the practical application of this construct is whether the modification of actin, either by labeling with rhodamine or by the nonspecific attachment of the peptide, interferes with normal actin polymerization. We tested this possibility with in vitro polymerization assays. The steady-state polymerization of actin was not affected by addition of the rhodamine label (Fig. 4A) nor by the formation of the disulfide linked FITC-TAT-Cys-S-STNB at Cys 374 of actin (FTSSAR, Fig. 4B, red trace). However, when DTT was added to reduce the SS bond, polymerization was dramatically inhibited (Fig. 4B, blue trace). Subsequent studies (Fig. 4C) in which actin was labeled with the TAT-peptide without fluorescein plus and minus DTT (Fig. 4C, red vs. black trace), indicated that only the fluorescein-labeled TAT peptide significantly inhibited actin polymerization.

Thus the dissociated FITC-TAT in cells, by adhering to actin filaments, made it difficult to determine, in the cells, if the

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**Fig. 4.** FTSSAR, but not Tat-SS-Actin-rhodamine (TSSAR), inhibits actin polymerization in vitro. Light scattering experiments to measure the actin polymerization. A: actin polymerization with rhodamine-actin; B: actin polymerization with FTSSAR, +/- DTT; C: actin polymerization with TSSAR +/- DTT. The latter two panels show the effects of SS bond reduction on polymerization.

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**Fig. 5.** TSSAR as a probe for actin polymerization in single freshly dissociated dVSMCs. Incorporation of TSSAR in the presence of 10^{-5} M phenylephrine (5 min) in freshly dissociated differentiated aorta smooth muscle cells. rhodamine stain, showing exogenous actin incorporation. Scale bar, 10 μm.
FITC-TAT had truly dissociated from the rhodamine-labeled actin. The FITC-TAT on the labeled actin in vitro also directly impairs actin polymerization but the protein transduction tag TAT by itself does not. The optimal construct for biological applications, then, is simply the nonfluorescently labeled TAT peptide conjugated to the rhodamine-labeled actin. Accordingly, we synthesized TAT-Cys-S-Rhodamine actin (referred to here as TSSAR) and added it to live, freshly enzymatically isolated cells in the presence of the α agonist phenylephrine to allow comparison to previous results (Fig. 5) and observed punctae of incorporated actin at the cortex of the cell. The punctae are indistinguishable from those we have previously reported to occur when G-actin is added to PE-stimulated permeabilized cells (11).

**DISCUSSION**

Here we report and validate a new method, which we have shown is suitable for use in intact differentiated cells for the direct monitoring of actin polymerization. Additionally, since we have previously shown that TAT-labeled peptides work well in whole vascular tissues (10), this new method is likely to also be successful in tissue work. This method utilizes a modified protein transduction tag from the HIV TAT sequence to allow the delivery of labeled G-actin into intact cells without disrupting the native state as occurs with cell permeabilization methods (5, 24).

Although actin dynamics are well known to play important roles in the maintenance of cell shape, cell division, cell migration, cell differentiation, endocytosis, and contractility in a variety of cell types (13, 18, 20), the study of actin dynamics in differentiated, not easily transfected, cells has been impeded by the lack of a suitable method for the introduction of labeled actin. In particular, the involvement of actin dynamics in the contractile functions of fully differentiated vascular smooth muscle cells has been reported only relatively recently in vascular smooth muscles and only directly visualized in permeabilized cells where important signaling molecules may be lost and intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]) is clamped constant (3, 15). Recently, our lab has reported evidence for the involvement in the regulation of contractility, of actin remodeling in the cortex of the dVSM cells. However, this could only be directly observed with the addition of labeled actin to permeabilized cells. In the present study, using TSSAR, we are able to directly confirm actin incorporation into the cortical cytoskeleton of intact dVSM cells under physiological conditions where cellular signaling pathways and [Ca$^{2+}$], dynamics are minimally affected.

Our initial goal was to synthesize a construct with G-actin as the cargo, coupled reversibly to the TAT-Cys-S-STNB peptide, allowing detachment of the peptide from the actin under the reducing conditions of the intracellular space. However, we were surprised to find that FITC-TAT-Cys can clearly inhibit actin polymerization in vitro. In fact it is possible that this peptide could have utility as a new cell permanent actin polymerization inhibitor when applied to tissue and animal models. However, we were also able to show that TSSAR causes no significant impairment of actin polymerization and hence can be used as a new tool to study actin dynamics in dVSMCs. Additionally, this method of actin delivery should be broadly applicable for the study of cytoskeletal dynamics in a range of differentiated cells and tissues.

**GRANTS**

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**REFERENCES**


