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Imaging Farnesyl Protein Transferase Using a Topologically Activated Probe

Wellington Pham,† Pamela Pantazopoulos, and Anna Moore*

Molecular Imaging Laboratory, Athinoula A. Martinos Center for Biomedical Imaging, Department of Radiology, Massachusetts General Hospital, Harvard Medical School, Room 2301, Building 149, 13th Street, Charlestown, Massachusetts 02129

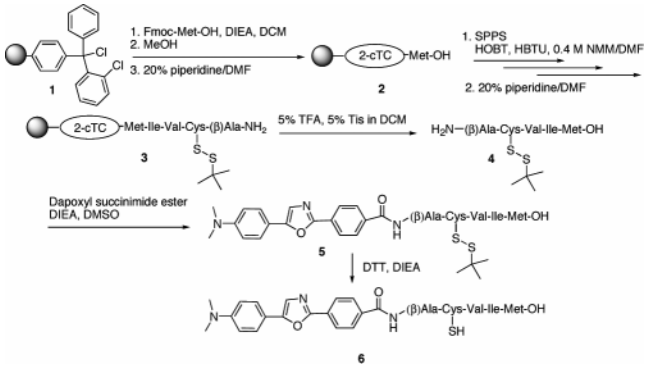
Received May 23, 2006; E-mail: amoore@helix.mgh.harvard.edu

Ras proteins play important roles in cell signal transduction, including the cell division cycle, programmed cell death, and differentiation.[1] In addition, Ras proteins are also responsible for about 30% of all human carcinomas,[2] including 90% of pancreatic, 50% of colon,[3] and 30% of lung[4] and breast cancers.[5] The proteins must be localized to the inner surface of the plasma membrane to be biologically active in all of these events. This first and most critical step in the post-translational modification is mediated by farnesyl protein transferase (FPT), which transfers a lipid farnesyl group from farnesyl pyrophosphate (FPP) to the cysteine sulfhydryl of Ras which ends with a CA_1A_2X motif at its carboxyl terminal.[6] Positions A_1 and A_2 are occupied by aliphatic amino acids, while X is held by either methionine or serine.[7] Since the relocation of Ras proteins from the cytoplasm to the plasma membrane upon farnesylation is crucial for tumor growth and survival, detection of FPT activity using molecular imaging probes based on a signal transduction platform is of considerable biomedical significance.

We report herein on the synthesis, characterization, and application of a fluorescent reporter probe to detect FPT in a cell-based assay. The underlying principle was to label the CA_1A_2X sequence with an environmentally sensitive fluorescent dye. Upon farnesylation by FPT, this sequence will be relocated from a hydrophilic (cytoplasm) to a hydrophobic (plasma membrane) environment. We expect the environmentally sensitive dye at this point to switch from a quenched to an enhanced fluorescent status. While screening a set of dyes for this purpose, we learned that dansyl and dapoxyl dyes are very environmentally sensitive. The fluorescence emissions of these dyes are strong in organic solvents and very weak in water. Detection of FPT by dansyl dye in a continuous assay has been pursued previously.[8] From the imaging point of view, dapoxyl dye has a number of distinct advantages over dansyl dye. First, dapoxyl dye has a high intensity absorption with a molar extinction of 26 000 M^{-1} cm^{-1} compared to 4000 M^{-1} cm^{-1} for dansyl dye. Second, dapoxyl dye could fluoresce with a λ_{max} at 600 nm, which may reduce background fluorescence from a biological environment. Third, dapoxyl dye has a very large Stokes shift; therefore, cross-talk resulting from scattered light is greatly reduced (optical spectra are available in Figure S1). Employing this environmentally activated dye in the system eventually offers a much more compact version compared to the previously reported fluorescence resonance energy transfer (FRET) probes[9] since only a single dye is employed, thus better enabling it for prying targets with minimum steric hindrance. We have assessed the topological effect of dapoxyl dye by imaging the signal enhancement in a hydrophobic environment using octanol (Figure S2).

To synthesize the probe, dapoxyl dye was selectively conjugated to the N-terminal of the Ki Ras-B peptide sequence of Cys-Val-Ile-Met (CVIM) via a β-alanine linker, while the thiol group on cysteine was protected by a *t*-buthiol moiety (Scheme 1). The stability of this protecting group over a mildly acidic environment enabled us to deprotect 2-chlorotrityl chloride (2-cTC) resin **1** in 5% TFA. To increase the efficiency of the synthesis, resin **1** was swelled carefully in DCM before coupling with the Fmoc-methionine manually. The product of methionylated 2-cTC **2** commenced the solid phase synthesis using a peptide synthesizer. After the synthesis, the Fmoc group was selectively deprotected from the N-terminal of β-alanine to provide **3**. The resin **1** was detached from the peptide in the presence of 5% TFA in DCM to provide **4**. After labeling the peptide with a dapoxyl dye, the *t*-buthiol group on compound **5** was deprotected by dithiothreitol (DTT) to provide **6**. The progress of the reaction was conveniently monitored by TLC in 9.5:0.5 of methylene chloride and methanol. The R_f spot could be visualized using an optical imaging system. In addition to this probe, we synthesized a native CVIM peptide **7**, which serves as a control using Wang resin (Supporting Information). To test the specificity of the labeled probe **6** compared to that of **7** toward FPT, we performed an FPT activity assay based on a reported procedure.[10,11] Furthermore, we used probe **5**, in which the farnesylation site was blocked by a *t*-buthiol protecting moiety as an additional control. As shown in Figure 1, FPT activity was reduced 7-fold in the labeled probe **6** (IC_{50} = 1.2 μM) compared to the unlabeled native CVIM **7** (IC_{50} = 0.17 μM). Meanwhile, farnesylation was abolished when the thiol moiety was blocked in compound **5** (IC_{50} = 2.4 mM). The binding constant (K_d) of probe **6** was calculated by measuring the fluorescence intensity of a fixed concentration of the probe while adding increasing concentrations of FPT. The data were analyzed as specified in Supporting Information, and the K_d was found to be 26 nM (Figure S3).

Scheme 1. Topologically Activated Probe



The results from the FPT assay confirm the selectivity of probe **6** and suggest the usefulness of its predecessor **5** as a control in a cell-based assay, given the challenge to find a control cell line that has no FPT expression. We performed a pure enzyme assay where probes **5** and **6** were incubated with FPT and FPP in a 96-well

† Current address: Vanderbilt University Institute of Imaging Science, 1161 21st Avenue South, Nashville, TN 37232. E-mail: wellington.pham@vanderbilt.edu.

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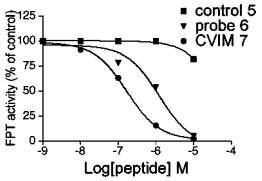

Figure 1. Farnesyl protein transferase activity. Value is an average of duplicate assays.

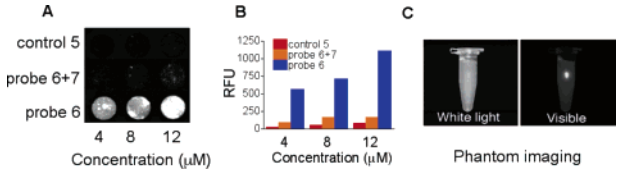

Figure 2. Live cell imaging of probe activation. Compounds were dissolved in DMSO and diluted 60-fold into the assay to give the final solvent concentration of <2%.

plate, and the fluorescence signal was detected by a high throughput plate reader. Farnesylation of probe **6** resulted in a 2-fold increase in the fluorescence signal compared to **5** (Figure S4, part A). Despite modest augmentation of fluorescence, we observed a significant and easily distinguished bright image of the probe ($p < 0.0001$) (Figure S4, part B). To demonstrate that farnesylation could result in fluorescent enhancement and enable imaging in a high throughput screening fashion in live cells, a human breast cancer cell line, MDA-MB-231, was incubated with either control **5** or probe **6** for 2 h. The medium was aspirated, and the cells were washed, trypsinized, and transferred to a 96-well plate for optical imaging as described in the Supporting Information. As expected, cells treated with probe **6** produced a remarkable fluorescence, compared to cells treated with the control **5** ($p < 0.0001$) (Figure 2A). In addition, we observed a reduced fluorescent signal when probe **6** was incubated with a 10-fold excess of peptide **7**, indicating specific interactions between probe **6** and the enzyme (Figure 2A). These observations suggest that there are two possibilities that turn the signal on. First, the attachment of the lipid in close proximity to the dye changes its environment. Second, the fatty acylated peptide could be relocalized to a hydrophobic plasma membrane. It is possible that both scenarios could take place. Quantitatively, for all three tested concentrations, probe **6** expressed significantly higher fluorescence signal (up to 30-fold, $p < 0.0001$) over the control at 4, 8, and 12 μM (Figure 2B). Collectively, these results strongly suggested that the fluorescent signal enhancement was indeed the outcome of a topological shift of the probe from an aqueous to a hydrophobic environment upon farnesylation. It is worth emphasizing that we observed a much stronger fluorescent enhancement in the cell-based assay compared to a pure enzyme assay. This difference could potentially arise from the availability of existing lipophilic membrane compartments inside the cell that were absent in the pure enzyme assay format. To further confirm the cellular activation of FPT and the potential future use of the probe in florescence-based applications, MDA-MB-231 cells were grown on a round cover slip to 70−80% confluency and incubated with control **5** or probe **6** for 2 h followed by fixing with 4% paraformaldehyde and counterstaining with DAPI for nuclear staining. As shown in Figure S5, the fluorescent signal coming from the cells treated with probe **6** was significantly brighter than that coming from the cells treated with control **5** under the same incubation conditions. To demonstrate the possibility that this technique could be utilized for tissue imaging, we performed imaging of a phantom imitating tumor in the tissue. To do that, human breast cancer cells MDA-MB-231 were incubated with probe **6** and injected into the middle of a tube filled with agarose gel. A bright fluorescent signal was detected in the middle of the tube after positioning the phantom in the optical imaging system (Figure 2C).

In conclusion, we report on the development and application of a novel method to image FPT in live cells using a topologically activated probe. Our results strongly suggest that the probe has good specificity toward FPT in in vitro assay, and farnesylation is a main switch that turns the fluorescent signal on upon incubation of breast cancer cells with probe **6**. This technique could have wide applications in high throughput drug screening and potentially in the imaging of small animal models.

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Supporting Information Available: Experimental details for the synthesis of probe **6** and control probe **7**, HPLC profiles of probes **5** and **6**, information regarding the FPT activity assays, cell-based assays, K_d calculation, and the complete ref 4. This material is available free of charge via the Internet at http://pubs.acs.org.

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