Supporting Information

for

A Protein Fluorescence Amplifier:
Continuous Fluorometric Assay for Rab Geranylgeranyltransferase

Yao-Wen Wu, Herbert Waldmann, Reinhard Reents, Frank H. Ebetino,
Roger S. Goody, and Kirill Alexandrov*
Synthesis of NBD-FPP

Synthesis and characterization of NBFPP was described elsewhere [1].

Figure S1. A) Synthesis of fluorescent diphosphates 7a and 7b a) NBD-Cl, NaHCO$_3$-buffer (pH 8-9), CH$_3$CN; 67-65%; b) PPTS, 60°C, EtOH; 93-94%; c) NCS, DMS, -30°C→0°C, 4a = quant.; 4b = quant; d) 5 , CH$_3$CN, rt, 3h; e) DOWEX ion exchange NBu$_4^+$/NH$_4^+$; 7a = 69%; 7b = 61%. B) Comparison of the fluorescent isoprenoid analogues with the native phosphoisoprenoids.

Determination of rate constants for mono- and double NBD-farnesylation

The in vitro HPLC assay for NBD-farnesylation and HPLC data processing was performed essentially as described previously for a geranylgeranylation assay [2]. In brief, 1 µM Rab7:REP1 and a mixture of 1 µM RabGGTase and 2 µM NBD-FPP were preincubated separately for 5 min at 25°C. The reaction was initiated by manual mixing of both components. After defined time intervals, 40 µL of reaction solution were withdrawn and quenched by addition of 80 µL 0.3% trifluoroacetic acid, and 20% glycerol followed by freezing in liquid nitrogen. For analysis, 100 µL of quenched reaction mix was subjected to reverse-phase chromatography. Samples were analyzed using a C4, 150 x 4.6 mm, 15 µm LUNA column (Phenomenx) driven by a Waters 600 s HPLC system. The column was equilibrated with 95% buffer A (0.1% trifluoroacetic acid in water) and 5% buffer B (0.1% trifluoroacetic acid in 100% acetonitrile).
at a flow rate of 1 ml/min. After injection and a 2-min wash step, proteins were eluted with a gradient from 5–70% of buffer B in 15 min, followed by elution for 8 min with 70% buffer B.

Figure S2. HPLC analysis of in vitro NBD-farnesylation reaction at defined time intervals.

As indicated in Figure S3, the peak of Rab7 (17.4 min) decreases, while the peaks of mono-NBD-farnesylated Rab7 (Rab7_NF) at 17.7 min and doubly NBD-farnesylated Rab7 (Rab7_NFNF) at 18.4 min increase. The primary HPLC data analysis was performed by integrating peak areas of absorbance at 280 nm for Rab7 and fluorescence intensity ($\lambda_{ex}$ = 490 nm, $\lambda_{em}$ = 535 nm) for mono- and double NBD-farnesylated Rab7.

Rate constants were extracted from time traces of Rab7, mono-NBD-farnesylated Rab7 (Rab7_NF), and doubly NBD-farnesylated Rab7 (Rab7_NFNF) using a global-fitting approach based on numerical integration according to a set of differential equations. Equations 1–6 follow. (See also Scheme S1.)

\[
\frac{d[Rab7]}{dt} = -k_1[Rab7][NBDFpp] + k_{-1}[Rab7:NBD{Fpp}] \\
\frac{d[\text{NBDFpp}]}{dt} = -k_1[Rab7][\text{NBDFpp}] + k_{-1}[Rab7:NBD{Fpp}] + k_s[Rab7\_NF][\text{NBDFpp}] - k_{-s}[Rab7\_NF : \text{NBDFpp}] \\
\frac{d[Rab7\_NF]}{dt} = k_s[Rab7\_NF][\text{NBDFpp}] - k_{-s}[Rab7\_NF : \text{NBDFpp}] \\
\frac{d[Rab7\_NFNF]}{dt} = k_s[Rab7\_NF][\text{NBDFpp}] - k_{-s}[Rab7\_NF : \text{NBDFpp}] - k_s[Rab7\_NF : \text{NBDFpp}] \]
The concentration of substrate, product, and intermediate were related via extinction coefficients and quantum yields, respectively: $A(Rab7) = Ec^*[Rab7] + Ec^*[Rab7:NBD-FPP]$; $F(Rab7_{NF}) = Qy^*[Rab7_{NF}] + Qy^*[Rab7_{NF}:NBD-FPP]$; $F(Rab7_{NFNF}) = 2*Qy^*[Rab7_{NF}]$. 

Figure S3. Kinetics and global fitting of unprenylated Rab7 (A), mono-prenylated Rab7 (B), and doubly prenylated Rab7 (C) in the in vitro NBD-farnesylation reaction.
This model resulted in an excellent fit using the program Scientis (MicroMath Software) giving $k_2 = 0.0026 \text{ s}^{-1}$ for the first prenyltransfer step, $k_4 = 0.002 \text{ s}^{-1}$ for the conversion of mono-NBD-farnesylated Rab7 into doubly NBD-farnesylated Rab7, and an effective (i.e. fitted) NBD-FPP concentration of 0.94 $\mu$M.

**Determination of the affinity of the mono-NBD-farnesylated Rab7:REP-1 complex toward RabGGTase**

Production and purification of mono-NBD-farnesylated Rab7:REP-1 complex was performed essentially as described but using the Rab7C207S single cysteine mutant and NBD-FPP as an isoprenoid donor. The product was characterized by MALDI-TOF and fluorescence scanning.

**Figure S4.** Titration of RabGGTase to a nominal concentration of 35 nM Rab7CSS_NF:REP-1 complex (excitation wavelength 479 nm, emission 525 nm) in presence (A) and absence (B) of 10 $\mu$M GGpp. Conditions: 25°C, 50 mM Hapes, pH7.2, 50 mM NaCl, 5 mM DTE. The solid line shows the fit to a quadratic equation and gives values of 382±38 nM and 368±6 nM for $K_d$ respectively.

**Determination of the dissociation rate constant of the mono-NBD-farnesylated Rab7:REP-1 complex from RabGGTase**
Figure S5. Stopped-flow measurement of the dissociation rate of the mono-NBD-farnesylated Rab7:REP1:RabGGTase complex: A) 35 nM of Rab7CSS_NF:REP-1 mixed with 35 nM RabGGTase complex was dissociated by addition of 400 nM Rab7:REP-1 in the presence of 2 µM NBD-FPP; B) 385 nM of Rab7CSS_NF:REP-1 was premixed with 3.3 µM RabGGTase and was dissociated by addition of 9.5 µM Rab7:REP-1. The interaction was monitored from the increase in fluorescence of the NBD group with excitation at 479 nm while fluorescence was recorded through a 530 nm cut-off filter. Mixing and measuring chambers were thermostated at 25°C. The transients were fitted to a single exponential function, resulting in the observed rate constants.

The observed rate constant of NBD-FPP incorporation into Rab7 determined by SDS-PAGE assay

The reaction rate constants obtained from the HPLC assay (Figure S3) were substantiated by SDS-PAGE based assay giving an observed rate constant for incorporation of NBD-FPP ($k_{obs}=0.0024$ s$^{-1}$).

For the reaction, 2 µM Rab7 and 2 µM REP-1 were incubated with 3 µM RabGGTase and 40 µM NBD-FPP in prenylation buffer at 25°C. At defined time intervals, 20 µL reaction solution were withdrawn and quenched by addition of 20 µL hot 2x SDS-PAGE sample buffer. The samples were boiled at 95°C for 3 min and 25 µL samples were loaded onto 15% SDS-PAGE. The fluorescent bands corresponding to the NBD-farnesylated protein were visualized in the gel using a Fluorescent Image Reader FLA-5000 (Fuji) (excitation laser: 473 nm, cut-off filter: 510 nm) followed by staining with Coomassie blue and scanning. The fluorescence intensities of the bands were quantitatively analyzed using AIDA densitometry software and the values were corrected for pipetting errors using Coomassie blue staining of the same
bands. The fit shown is to a single-exponential equation, giving an observed rate constant $k_{obs} = 0.0024 \pm 0.0003$ s$^{-1}$.

**Figure S6.** SDS-PAGE based assay for in vitro NBD-farnesylation of Rab7.

**Observed rate constants of mono-NBD-farnesylation of Rab7 single cysteine mutants**

**Figure S7.** Single turnover NBD-farnesylation of Rab7C207S and Rab7C205S with continuous fluorescence assay and SDS-PAGE assay. A) In the fluorescence assay, the reaction contained 200 nM of REP-1, GGTaseII and 4 µM NBD-FPP and was initiated with 200 nM Rab7 in assay buffer (50 mM HEPES, pH7.2, 50 mM NaCl, 5 mM DTE, 2 mM MgCl$_2$, 10 µM GDP) at 25°C. ($\lambda_{ex}=479$ nm, $\lambda_{em}=521$ nm) The solid lines represent fits to single-exponential
functions for Rab7C207S in red ($k_{\text{obs}} = 0.00084 \text{ s}^{-1}$) and for Rab7C205S in blue ($k_{\text{obs}} = 0.00027 \text{ s}^{-1}$). B) 4 µM Rab7 and 4 µM REP-1 were incubated with 4 µM of RabGGTase and 50 µM NBD-FPP in the same prenylation buffer at 25°C. At defined time intervals, 10 µL samples were withdrawn and quenched by addition of 10 µL 2x SDS-PAGE sample buffer. The samples were boiled at 95°C for 3 min and were loaded onto 15% SDS-PAGE. The fluorescent bands corresponding to the NBD-farnesylated protein were analyzed as described above. The solid lines represent fits to single-exponential functions for Rab7C207S in red ($k_{\text{obs}} = 0.00083 \text{ s}^{-1}$) and for Rab7C205S in blue ($k_{\text{obs}} = 0.00028 \text{ s}^{-1}$).

**Scheme S2.** The kinetic and equilibrium constants of the Rab7 prenylation reaction using geranylgeranyl pyrophosphate (black) or NBD-farnesyl pyrophosphate (red) as a substrate.

**Fluorescence assay**

Data were collected on a Spex FluoroMax-3 spectrofluorimeter (Jobin Yvon Inc., Edison, NJ) with $\lambda_{\text{ex}} = 479 \text{ nm}$ (slit width = 1 nm) and $\lambda_{\text{em}} = 520 \text{ nm}$ (slit width = 5 nm). All spectra were obtained at 37°C in a thermostatted cuvette holder. Empty cuvettes were prewarmed in the thermostatted holder for 5 min. The assay components (RabGGTase, REP, Rab7) were assembled and the mixture was preincubated at 37°C before the reaction was initiated by the addition of NBD-FPP. The contents were quickly mixed by flicking the tube, and a 400 µL sample was pipetted into the prewarmed cuvette. Rates were derived from the initial linear part (less than 10 min) of the progress curve. Conversion of the reaction velocities from fluorescence intensity per unit time to concentration per unit time was performed as shown in Equation 8, where $\varepsilon$ is the enhancement factor by which the substrate quantum yield is enhanced after converting to product and conversion factor $m$ is the slope of the line.
generated in a plot of concentration of mono-NBD-farnesylated Rab7:REP-1 versus fluorescence intensity.

\[
V(\mu M \cdot s^{-1}) = \left[ V \left( \frac{\text{fluorescence}}{s} \right) \right] \left[ \frac{1}{m \left( \frac{\mu M}{\text{fluorescence}} \right)} \right] \left( e \right) \left( e - 1 \right)
\]  

(8)

**Figure S8.** Determination of \(K_m\) for Rab7:REP-1 and NBD-FPP. A) Reaction mixture contained 10 \(\mu M\) NBD-FPP, 0.02 \(\mu M\) RabGGTase and indicated concentrations of Rab7:REP-1 complex. B) Reaction mixture contained 2 \(\mu M\) Rab7:REP-1 complex, 0.02 \(\mu M\) RabGGTase and indicated concentrations of NBD-FPP. Solid lines show the fits to the Michaelis-Menten equation.
**Figure S9.** Inhibition of RabGGTase by NE10790. The reaction mixture contained 4 µM NBD-FPP, 0.02 µM RabGGTase and varying concentrations of Rab7:REP-1 complex in the absence and presence of indicated concentrations of inhibitor.

**Determination of IC\textsubscript{50} value for NE10790 mediated inhibition of rab prenylation**

Concentration dependent inhibition of RabGGTase by NE10790 was performed under the conditions: 37°C, pH 7.2, 20 nM RabGGTase, 2 µM Rab7:REP1, 10 µM NBD-FPP, in the presence of increasing concentrations of inhibitor. IC\textsubscript{50} was determined with equation below and gave the value of 502±113 µM.

\[
\frac{v_i}{v_0} = \frac{100\%}{1 + \left(\frac{[I]}{IC_{50}}\right)^s}
\]

Where \(v_i\) is the inhibited velocity, \(v_0\) is the uninhibited velocity, \([I]\) is the inhibitor concentration, \(s\) is a slope factor and IC\textsubscript{50} is the concentration of the inhibitor that produces 50% inhibition. The lower data limit is 0, ie the data are background corrected, and the upper data limit is 100, that is, the data are range corrected.

**References**

