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Phosphonocarboxylates Inhibit the Second Geranylgeranyl Addition by Rab Geranylgeranyl Transferase*

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Rab geranylgeranyl transferase (RGGT) catalyzes the post-translational geranylgeranyl (GG) modification of (usually) two C-terminal cysteines in Rab GTPases. Here we studied the mechanism of the Rab geranylgeranylation reaction by bisphosphonate analogs in which one phosphonate group is replaced by a carboxylate (phosphonocarboxylate, PC). The phosphonocarboxylates used were 3-PEHPC, which was previously reported, and 2-hydroxy-3-imidazo[1,2-*a*]pyridin-3-*yl*-2-phosphonopropionic acid ((+)-3-IPEHPC), a >25-fold more potent related compound as measured by both IC_{50} and K_i. (+)-3-IPEHPC behaves as a mixed-type inhibitor with respect to GG pyrophosphate (GGPP) and an uncompetitive inhibitor with respect to Rab substrates. We propose that phosphonocarboxylates prevent only the second GG transfer onto Rabs based on the following evidence. First, geranylgeranylation of Rab proteins ending with a single cysteine motif such as C*AAX*, is not affected by the inhibitors, either *in vitro* or *in vivo*. Second, the addition of an *-AAX* sequence onto Rab-CC proteins protects the substrate from inhibition by the inhibitors. Third, we demonstrate directly that in the presence of (+)-3-IPEHPC, Rab-CC and Rab-C*X*C proteins are modified by only a single GG addition. The presence of (+)-3-IPEHPC resulted in a preference for the Rab N-terminal cysteine to be modified first, suggesting an order of cysteine geranylgeranylation in RGGT catalysis. Our results further suggest that the inhibitor binds to a site distinct from the GGPP-binding site on RGGT. We suggest that phosphonocarboxylate inhibitors bind to a GG-cysteine binding site adjacent to the active site, which is necessary to align the mono-GG-Rab for the second GG addition. These inhibitors may represent a novel therapeutic approach in Rab-mediated diseases.

Most proteins of the Ras-like GTPase superfamily need to be post-translationally modified by prenyl groups to associate with cellular membranes and to activate downstream effectors (1). Protein prenylation involves the formation of a thioether link between conserved C-terminal cysteines in protein substrates and farnesyl pyrophosphate (FPP)[7] or geranylgeranyl pyrophosphate (GGPP) (2, 3). These prenyl pyrophosphates, which originate from the mevalonate pathway are utilized by three different prenyltransferase enzymes (2, 3). Farnesyl transferase (FT) and geranylgeranyl transferase type I (GGT-I) transfer FPP or GGPP, respectively, to a cysteine residue in the context of a C-terminal C*AAX* motif, where C is a cysteine, *A* is an aliphatic residue, and *X* is any amino acid. *X* contributes significantly to substrate specificity in FT and GGT-I (2). Rab geranylgeranyl transferase (RGGT) is distinct from FT and GGT-I in that it specifically recognizes Rab proteins, which vary in their C terminus containing C*CXX*, *XCXC*, *XXCC*, C*CXXX*, C*AAX*, and other motifs (3). RGGT exhibits exquisite specificity for Rabs due to the strict requirement of Rab escort protein (REP), which is a general Rab-GDP binding protein (4). RGGT is a heterodimeric enzyme consisting of a 60-kDa α-subunit and a 38-kDa β-subunit, and shares 30% homology with its FT and GGT-I counterparts (5). The α-subunit associates with REP in a very small area compared with the entire surface occupied by the complex, whereas the β-subunit binds one molecule of GGPP in a large hydrophobic cavity (5, 6).

Although a large number of specific FT and GGT-I inhibitors have been developed as potential anti-cancer agents (7, 8), few inhibitors of RGGT exist. Recently the phosphonocarboxylate 3-PEHPC was identified as a specific RGGT inhibitor, thereby selectively preventing the prenylation of Rab proteins in cells (9,

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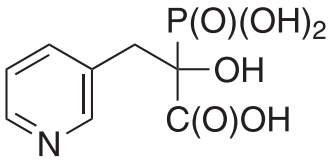
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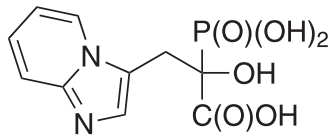
[7] The abbreviations used are: FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; REP, Rab escort protein; 3-PEHPC, 2-hydroxy-2-phosphono-3-pyridin-3-*yl*-propionic acid; 3-IPEHPC, 2-hydroxy-3-imidazo[1,2-*a*]pyridin-3-*yl*-2-phosphonopropionic acid; HPLC, high performance liquid chromatography; PC, phosphonocarboxylate; ICMT, isoprenyl cysteine methyltransferase; FT, farnesyl transferase; GGT-I, geranylgeranyl transferase type I; RGGT, Rab geranylgeranyl transferase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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1, 3-PEHPC

STRUCTURE 1



2, 3-IPEHPC

STRUCTURE 2

10). This compound was derived from the bisphosphonate, risedronate, which is used clinically in the treatment of osteoporosis, due to its ability to potently inhibit the activity of bone-resorbing osteoclasts (11). Although bisphosphonates such as risedronate prevent Rab prenylation, they do not act by inhibiting RGGT. Rather, these drugs prevent the synthesis of FPP and GGPP by inhibiting FPP synthase, thereby preventing all protein prenylation (12). 3-PEHPC is a weak RGGT inhibitor and consequently a weak inhibitor of bone resorption. However, we have recently identified a similar phosphonocarboxylate, (+)-3-IPEHPC, which is at least 25 times more potent than 3-PEHPC as an inhibitor of RGGT. Here, we report the surprising finding that phosphonocarboxylate inhibitors of RGGT act by inhibiting only the second Rab geranylgeranylation event, therefore providing specificity toward Rab proteins with a double cysteine C-terminal motif.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Human REP1 cDNA was cloned into pFastBacHTb and produced as described previously (13). Rat RGGT was prepared as described previously (13). Canine Rab1a, human Rab27a, human Rab5a, human Rab18, and human Rab6a were amplified by PCR and cloned into pET14b and human Rab13 and mouse Rab23 were cloned into pGEX-4T-1 as described previously (14). The indicated C-terminal sequence mutants were obtained by using the QuikChange mutagenesis kit (Stratagene). The sequences of all plasmid constructs used were confirmed by DNA sequencing.

Recombinant Proteins—The recombinant proteins RGGT and REP1 were prepared by infection of Sf9 cells with recombinant baculoviruses encoding each subunit of the desired enzyme and purified by nickel-Sepharose affinity chromatography as described previously (13, 15). Recombinant His_6 and glutatathione *S*-transferase-tagged Rab proteins were purified by nickel-Sepharose or glutathione-Sepharose affinity as described previously (16, 17). All recombinant proteins were snap frozen in small aliquots and stored at −80 °C until use.

Bisphosphonates—2-Hydroxy-2-phosphono-3-pyridin-3-*yl*-propionic acid (3-PEHPC, previously referred to as NE-10790) (Structure 1) was a gift from Procter & Gamble Pharmaceuticals. 2-Hydroxy-3-imidazo[1,2-a]pyridin-3-*yl*-2-phosphonopropionic acid (3-IPEHPC) (Structure 2) was synthesized and resolved into its component enantiomers by methods reported elsewhere.[8] The (+)-enantiomer was used in the present study, because this has been shown to be selective toward RGGT, and a more potent inhibitor of this enzyme than the (−)-enantiomer.[8] The enantiopurity was determined to be >98% by chiral HPLC using a Prontosil AX QN (eluted isocratically with 0.7 M TEAA buffer containing 75% MeOH at pH 5.8; UV detection at 265 nm, flow rate 3 ml/min).[8] The more active (+)-enantiomer elutes first. The specific optical rotation $[\alpha]_D^{25}$ +109° was measured on a Jasco J100 Polarimeter (Structures 1 and 2).

In Vitro Prenylation Assay—RGGT activity was measured by determining the amount of [^{3}H]GGPP transferred to Rab proteins (15, 18). Unless otherwise indicated, the standard reaction mixture contained the following concentrations in a final volume of 25 μl: 50 mM sodium HEPES (pH 7.2), 5 mM $MgCl_2$, 1 mM Nonidet P-40, 1 mM dithioerythritol, 5 μM [^{3}H]GGPP (specific activity = 800 dpm/pmol), 4 μM Rab proteins, 2 μM REP1, and 50 nM RGGT. After 20 min at 37 °C, the reaction was stopped by addition of 1 ml of ethanol/HCl (9:1) and the incorporated radioactivity was measured by filtration on glass fiber followed by scintillation counting (18).

Kinetic Analyses—For the inhibition pattern experiments with biphosphonate compounds, data were collected for times where product accumulation was linear. The observed initial velocity data were fitted to Equation 1 for mixed-type inhibition, where K_m is the Michaelis constant for the varied substrate S, and K_i is the mixed-type inhibition constant for (+)-3-IPEHPC and 3-PEHPC.

$$\frac{1}{v} = \frac{K_m}{V_{max}}\left(1 + \frac{[I]}{K_i}\right)\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{\alpha K_i}\right) \quad \text{(Eq. 1)}$$

For uncompetitive inhibition the observed initial velocity data were fitted to Equation 2.

$$\frac{1}{v} = \frac{K_m}{V_{max}}\frac{1}{[S]} + \frac{1}{V_{max}}\left(1 + \frac{[I]}{K_i}\right) \quad \text{(Eq. 2)}$$

The K_i and associated S.E. for (+)-3-IPEHPC and 3-PEHPC were calculated using shared parameter curve fitting for all inhibitor concentrations using the average of duplicate determinations. The error represents the divergence between fitted curves.

Proteolytic Digestion of Prenylated Rab Proteins and Peptide Analysis—The peptides were prepared essentially as described previously (19, 20). The RGGT mixture (25 μl), 2 μM REP1, 50 nM RGGT, 5 μM Rab proteins, 5 μM GGPP (specific activity = 1600 dpm/pmol) was precipitated by addition of cold acetone (1 ml) for 1 h at 4 °C. The pellet was washed with 1 ml of cold acetone and resuspended in 100 μl of 25 mM Tris-HCl (pH 8), 4% CHAPS, 1 mM EDTA, and 3 μg of endoproteinase C-Lys (Roche Applied Science) and incubated overnight at 37 °C. The resulting peptides were isolated on a reverse-phase μRPC

[8] C. E. McKenna, F. Coxon, I. Mallard-Favier, K. M. Blazewska, J. M. Bala, M. W. Lundy, F. H. Ebetino, R. A. Baron, M. C. Seabra, M. S. Marma, B. A. Kashemirov, and M. Rogers, manuscript in preparation.

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C2/C18 SC 2.1/10 column (GE Healthcare) using a SMART System chromatography instrument (GE Healthcare). The column was equilibrated in MilliQ water with 0.06% trifluoroacetic acid at room temperature at a flow rate of 200 μl/min. 50 μl of sample were injected and the peptides were separated by a 3.5-ml linear gradient (0–70% acetonitrile) and 2.5 ml of 100% acetonitrile (0.056% trifluoroacetic acid). The radioactivity in each fraction (200 μl) was measured by scintillation counting (Beckman LS 6000SC).

[^{3}H]GGPP Binding to RGGT—The reaction mixture (25 μl), 2 μM REP1, 100 nM RGGT, 4 μM Rab proteins, 2 μM [^{3}H]GGPP (specific activity = 4500 dpm/pmol), and 100 μM (+)-3-IPEHPC were incubated for 20 min at 37 °C and mixed with 2 μl of rabbit anti-RGGT (H492) plus 25 μl of protein G beads (GE Healthcare). The solution was gently shaken for 2 h at 4 °C. The beads were washed 4 times with 1 ml of phosphate-buffered saline and the proteins were precipitated by adding 200 μl of EtOH/HCl (9:1). The samples were centrifuged at 20,000 × *g* for 10 min and the radioactivity in 200 μl of supernatant was determined by scintillation counting.

Analysis of Prenylation of GFP-Rabs in 293 Cells—Human embryonic kidney 293 (293) cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and seeded into 12-well plates at 2 × 10^5 cells/well. The following day, cells were transfected with 400 ng of purified plasmids (the epidermal growth factor protein-Rab constructs detailed above) using FuGENE 6 transfection reagent, according to the manufacturer's instructions (Roche). Immediately after transfection, 0–1.5 mM 3-PEHPC was added to the culture medium and cells incubated for 24 h. Cells were then lysed in Triton X-114 fractionation buffer, and prenylated Rabs separated from unprenylated Rabs as previously described (9). 30 μg of fractionated lysates (aqueous and detergent-rich phases, containing unprenylated and prenylated Rabs, respectively) were electrophoresed on 12% polyacrylamide Criterion gels (Bio-Rad), transferred to polyvinylidene difluoride membrane, then Western blotted for GFP-Rabs using an anti-GFP antibody and also β-actin as a loading control (Sigma). Bands were visualized following hybridization with secondary antibodies conjugated to Alexa Fluor-680 and Alexa Fluor-800 using an Odyssey infra red imaging system (LiCor).

Analysis of Localization of GFP-Rabs in 293 Cells—293 cells were seeded onto 9-mm glass coverslips in 48-well plates at 4 × 10^4 cells/well, then transfected the following day with 100 ng of epidermal growth factor protein-Rab constructs using FuGENE 6. In some cases, cells were also incubated with 1 mM 3-PEHPC. Cells were incubated for 24 h, then fixed in 4% formaldehyde. Nuclei were counterstained with TO-PRO-3 iodide and the cells were examined using a Zeiss LSM510 META confocal microscope after mounting the coverslips onto glass slides.

Isoprenyl Cysteine Methyltransferase (ICMT) Assay—Enriched ICMT membranes were a kind gift of Patrick J. Casey (Department of Pharmacology, Duke University). The methylation of Rab1a was measured after *in vitro* prenylation of the protein. The final volume on prenylation reaction (25 μl) contains: 50 mM sodium HEPES (pH 7.2), 5 mM $MgCl_2$, 1 mM dithioerythritol, 20 μM cold GGPP, 10 μM Rab1a proteins, 2 μM REP1, 100 nM RGGT, and 100 μM (+)-3-IPEHPC. After 30 min at 37 °C, 5 μl of buffer (50 mM sodium HEPES (pH 7.2), 5 mM $MgCl_2$, 1 mM dithioerythritol, 10 μM [^{3}H]*S*-adenosylmethionine (specific activity = 650 dpm/pmol)) and 1 μg of enriched ICMT membranes were added for 40 min. The reaction was stopped and treated as described previously.

TABLE 1

IC_{50} values for RGGT inhibition by (+)-3-IPEHPC and 3-PEHPC

The values represent the mean ± S.E. determined from duplicate determinations of at least two independent experiments.

Rab substrate	IC_{50}	
	(+)-3-IPEHPC	3-PEHPC
	μM	
Rab1a-CC (WT)	1.27 ± 0.24	31.85 ± 2.13
Rab1a-CSC	1.11 ± 0.30	ND[a]
Rab1a-CS	221.25 ± 11.49	>2000
Rab1a-SC	187.82 ± 8.30	>2000
Rab1a-CCS	0.91 ± 0.25	ND
Rab27a-CGC (WT)	0.83 ± 0.50	32.68 ± 1.95
Rab27a-CVLS	>800	>2000
Rab5a-CCSN (WT)	0.43 ± 0.06	43.47 ± 9.85
Rab5a-CCQNI	16.52 ± 4.42	>2000
Rab5a-CCVLL	5.91 ± 0.50	860 ± 80
Rab5a-CVLL	>800	>2000
Rab6a-CSC	27.22 ± 2.28	1592 ± 95
Rab13-CSLG (WT)	>800	>2000
Rab18-CSVL (WT)	>800	>2000
Rab23-CSVP (WT)	>800	>2000

[a] ND, not determined.

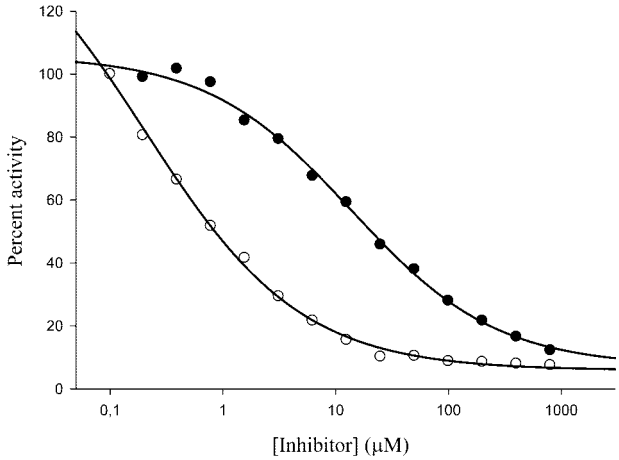


FIGURE 1. **Inhibition of RGGT activity by phosphonocarboxylates.** Final concentrations for the reaction mixture are REP1 (2 μM), RGGT (50 nM), GGPP (5 μM), Rab1a (4 μM) and increasing concentrations of (+)-3-IPEHPC (○) and 3-PEHPC (●). The reactions were incubated for 20 min at 37 °C. The values represent the means determined from duplicate determinations of two independent experiments. This experiment is representative of two other independent experiments.

RESULTS

PCs Are Mixed-type Inhibitors of RGGT—We previously reported weak RGGT inhibition by a racemic phosphonocarboxylate compound, 3-PEHPC (10). The pyridine cycle of this compound was replaced by an imidazo[1,2-a]pyridine cycle to create (+)-3-IPEHPC, a PC analog of the bisphosphonate, minodronate. We found that (+)-3-IPEHPC was 25-fold more potent for RGGT inhibition compared with 3-PEHPC using Rab1a as the substrate (IC_{50} (+)-3-IPEHPC = 1.3 ± 0.22 μM; IC_{50} 3-PEHPC = 31.9 ± 2.1 μM; Table 1 and Fig. 1). To assess the inhibition type with respect to both substrates, Rab and

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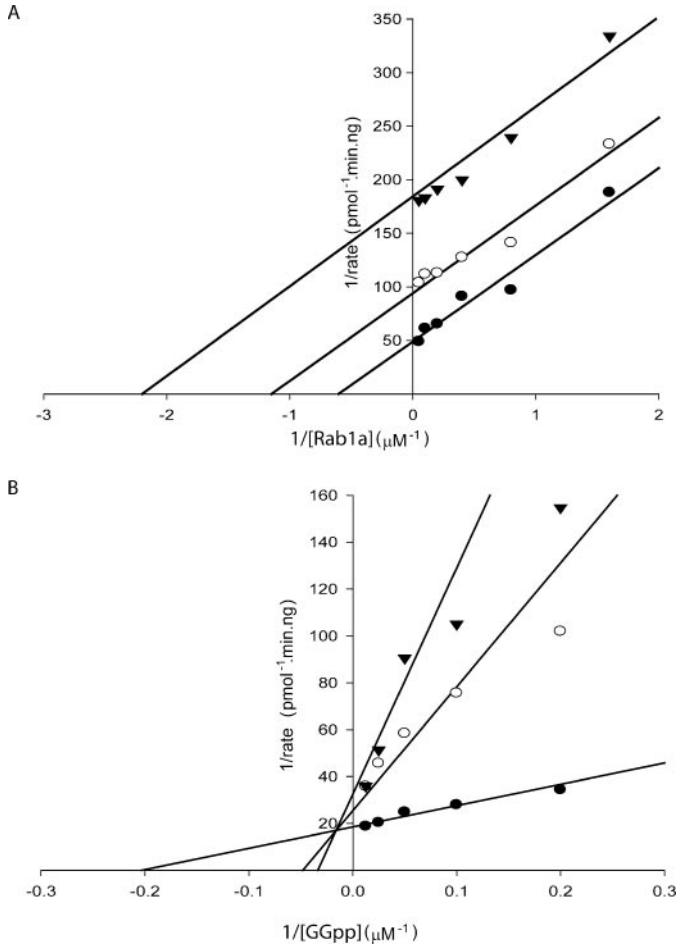


FIGURE 2. **Characterization of the inhibition of RGGT by (+)-3-IPEHPC.** *A*, Lineweaver-Burk plot for the inhibition of RGGT by (+)-3-IPEHPC when Rab1a was the varied substrate. Prenylation assays (see "Experimental Procedures") were carried out in the presence of a fixed concentration of GGPP substrate (5 μM) and the indicated concentrations of Rab1a at 0 (●), 0.75 (○), or 2 μM (▼) (+)-3-IPEHPC. Data were fit to Equation 1. *B*, Lineweaver-Burk plot for the inhibition of Rab1a prenylation when GGPP was the varied substrate. RGGT assays (see above) were carried out in the presence of a fixed concentration of Rab1a substrate (4 μM) and the indicated concentrations of GGPP at 0 (●), 0.5 (○), or 1 μM (▼) (+)-3-IPEHPC. Data were fitted to Equation 1. For both panels, data represent the means of duplicate determinations from a single experiment that is representative of three such experiments.

GGPP, we designed kinetic experiments using (+)-3-IPEHPC. Double-reciprocal plots obtained from such experiments are shown in Fig. 2*A* for Rab and Fig. 2*B* for GGPP. Equations describing competitive, noncompetitive, uncompetitive, and mixed-type inhibitions were fitted to the data for Rab1a, resulting in a best fit for an uncompetitive inhibition (Fig. 2*A*); the calculated K_i under the conditions employed in this assay was 0.21 ± 0.09 μM. Similar analysis for the lipid substrate, GGPP, suggests a mixed-type inhibition, *i.e.* the inhibitor behaves both as competitive and noncompetitive inhibitor (Fig. 2*B*); we calculated that K_i = 0.074 ± 0.029 μM. Interestingly, 3-PEHPC gave a similar type of inhibition for both substrates with K_i values of 5 ± 0.18 and 33.6 ± 11.1 μM for GGPP and Rab1a substrates, respectively (Table 2).

Inhibition of Rab Prenylation by PCs Is Dependent on the C-terminal Prenylation Motif—The velocity of the prenylation reaction can be described as $v_t = v_1 \times v_2$, where v_t is the total velocity of the reaction and v_1 and v_2 the velocities for the addition of the first and second GGPP, respectively. To assess the possibility that different forms of the enzyme-substrate complex are unequally sensitive to (+)-3-IPEHPC, we measured IC_{50} values for Rab1a substrates with different C-terminal motifs. To generate mono-prenylated or di-prenylated products at different C-terminal positions, the -CC motif of Rab1a was replaced by CSC, CCS, CS, or SC, and the respective recombinant proteins were produced as His-tagged fusion proteins in *Escherichia coli* and purified. *In vitro* prenylation assays were then performed with those substrates at different inhibitor concentrations (Table 1). The IC_{50} values generated for Rab1a-CC, Rab1a-CSC, and Rab1a-CCS proteins were very similar, at around 1 μM. This result suggests that different double-cysteine motifs in the context of the same Rab does not affect significantly the inhibition by (+)-3-IPEHPC. Conversely, the IC_{50} varied with the Rab substrate used. Comparing different Rab proteins containing double cysteine motifs, Rab1a (CC), Rab5a (CCSN), Rab6a (CSC), and Rab27a (CGC), we observed a 30-fold higher IC_{50} for Rab6 as compared with Rab1a, Rab5a, and Rab27a (Table 1). Similar differences were found when we measured IC_{50} values for 3-PEHPC, which was 50-fold higher for Rab6 substrate compared with Rab27a.

TABLE 2

Experimental kinetic constants for RGGT inhibition by (+)-3-IPEHPC and 3-PEHPC

The values represent the means ± S.E. determined from duplicate determinations of three independent experiments.

Inhibitor	Substrate	
	Rab1a	GGPP
(+)-3-IPEHPC	Uncompetitive K_i = 0.211 ± 0.091 μM	Mixed-type K_i = 0.074 ± 0.029 μM
3-PEHPC	Uncompetitive K_i = 33.56 ± 11.05 μM	Mixed-type K_i = 5 ± 0.18 μM

We next determined whether other factors in the C-terminal motif affected the inhibitory properties of PCs. In the context of Rab5a (CCSN), changing to -CCQNI (present in Rab11a), resulted in an increase in IC_{50} from 0.4 to 16.5 μM for (+)-3-IPEHPC (Table 1), whereas when the motif was changed to CCVLL, the IC_{50} was increased to a lesser extent (IC_{50} = 5.9 μM) (Table 1). Similar differences were found using (+)-3-IPEHPC as the inhibitor (Table 1). Both C-terminal modified Rab5 proteins were doubly geranylgeranylated as verified by chromatography as described below (data not shown). The spatial alignment of the CCVLL and CCQNI sequences showed high similarity in terms of surface area and length, but the CCQNI sequence was much more hydrophilic than CCVLL (the grand average of hydropathy coefficients or GRAVY are 0.5 and 3.36, respectively). Therefore, the length and the polarity of the C-terminal prenylation motif is an important factor in influencing the inhibitory properties of PCs.

Surprisingly, (+)-3-IPEHPC no longer behaved as an inhibitor when single cysteine Rabs were used in the assay. We studied three wild-type proteins, Rab13 (CSLG, as a glutatathione *S*-transferase fusion), Rab18 (CSVL, as a glutatathione *S*-transferase fusion), and Rab23 (CSVP), and in all three cases no significant inhibition was obtained with (+)-3-IPEHPC concentrations of up to 800 μM (Table 1). Consistently, both Rab5a (Rab5aCVLL) and Rab27a (Rab27aCVLL) single cysteine

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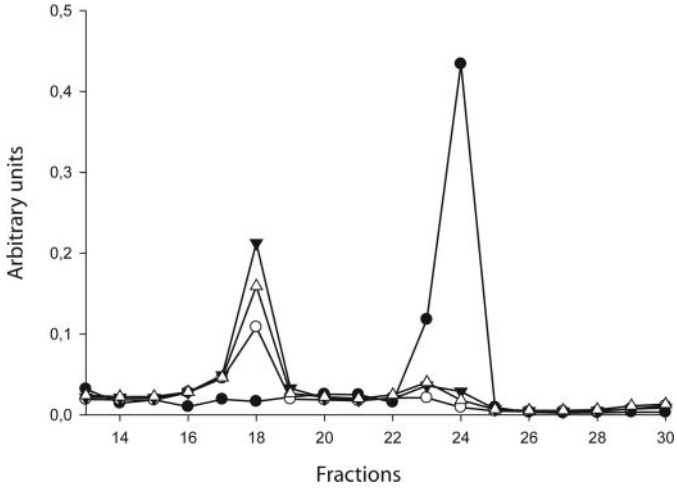


FIGURE 3. **C18 reverse-phase HPLC analysis of [^{3}H]GG-labeled Rab1a tryptic peptides.** Rab1a-CC (●), Rab1a-CS (○), Rab1a-SC (▼), and Rab1a-CC + (+)-3-IPEHPC (100 μM) (△) were incubated separately with [^{3}H] GGPP and RGGT/REP1, and digested with endoproteinase C-Lys. The respective digestion mixtures were then purified by reverse-phase HPLC (μRPC C2/C18 SC 2.1/10 column) using a SMART system, and the radioactivity of each fraction was plotted *versus* the eluted fractions. We observed similar results when the reaction mixture was digested with trypsin. This experiment is representative of three independent experiments.

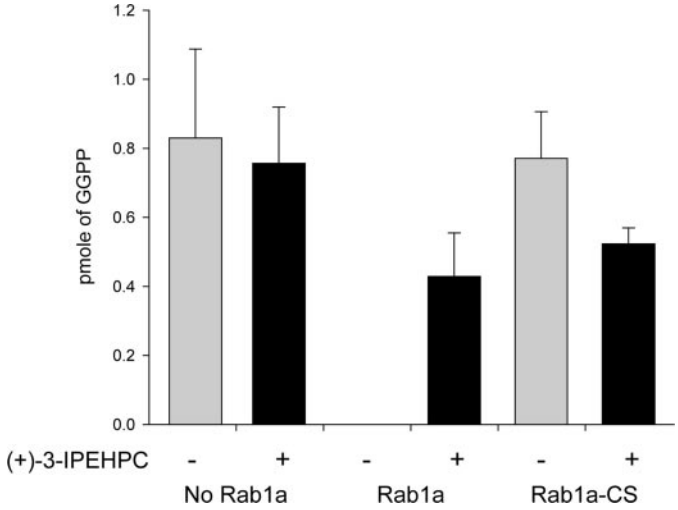


FIGURE 4. **GGPP binding to RGGT.** The prenylation mixture (20 μl) contains REP1 (2 μM), RGGT (100 nM), Rab1a (4 μM) substrate, and GGPP (2 μM), and as indicated on the graph 100 μM (+)-3-IPEHPC. After 30 min at 37 °C, 2 μl of polyclonal anti-RGGT (H492) and 25 μl of protein G beads were added to the reaction mixture and gently agitated for 2 h at 4 °C. The beads were washed with phosphate-buffered saline and the immunocomplexes were precipitated. GGPP molecules associated with RGGT were recovered in the supernatant and detected by scintillation counting. Background of the reaction (solution without RGGT) was removed from each observed condition. Data represent the mean of four different experiments.

mutants showed the same pattern (Table 1). Furthermore, Rab1a mutants with a single prenylatable cysteine (Rab1a-CS and Rab1a-SC) exhibited a 100-fold increase in IC_{50} values for (+)-3-IPEHPC when used as substrates (221 ± 11 and 187 ± 8 μM, respectively) as compared with the wild-type Rab1a-CC (Table 1). These results indicate that the C-terminal sequence of the Rab substrates plays a critical role in determining susceptibility to inhibition by PCs, with the mono-geranylgeranylated (mono-GG) Rab proteins unaffected, or only partially affected by the compound, and suggest that the first event of prenylation (v_1) is not affected by the inhibitor. This model could also explain why (+)-3-IPEHPC behaved as a mixed-type inhibitor. The compound may act differently for each prenylation reaction, v_1 and v_2, *i.e.* the different transition states for single and double prenylation.

PCs Inhibit Only the Second Event of Prenylation—To test our hypothesis that only the second prenylation event is inhibited by (+)-3-IPEHPC, we developed a chromatographic method to discriminate between mono-geranylgeranylated and di-geranylgeranylated forms of Rab proteins. Initially, we defined the elution volumes of mono-geranylgeranylated and di-geranylgeranylated peptides. We subjected Rab1a-CC, Rab1a-CS, and Rab1a-SC to *in vitro* prenylation and the products of the reaction were digested by endoproteinase C-Lys. The peptides obtained were separated on a C2/C18 reverse phase column with an acetonitrile/water gradient and the elution of [^{3}H]GGPP-labeled fractions was analyzed (Fig. 3). Under the conditions of the experiment, a clear separation was achieved between mono- and di-geranylgeranylated peptides. The Rab1a-CC digestion profile showed a single peak eluting at 95% acetonitrile (fraction 24), whereas the Rab1a-CS and SC peptides eluted as a single peak at 75% acetonitrile (fraction 18) (Fig. 3). Then, we performed *in vitro* prenylation reactions with Rab1a-CC in the presence or absence of (+)-3-IPEHPC at a saturating concentration (100 μM). Strikingly, in the presence of (+)-3-IPEHPC, the di-geranylgeranylated peak disappeared, whereas a peak corresponding to mono-geranylgeranylated peptide appeared (Fig. 3). Moreover, an identical profile was obtained when Rab1a-CSC or Rab5 (CCSN) were used as substrates, as well as when the proteins were digested with trypsin instead of endoproteinase C-Lys (data not shown). Furthermore, we detected mono-GG proteins when RGGT was treated with 3-PEHPC, similar to the results obtained with (+)-3-IPEHPC (data not shown). These results clearly indicate that only the second prenylation event is inhibited by PC compounds, and that the C-terminal ending of Rab proteins is a critical determinant of susceptibility to inhibition.

(+)-3-IPEHPC Does Not Preclude GGPP Binding to RGGT—Based on the competition experiments, the inhibitor should not compete for substrate binding to RGGT, or only partially in case of GGPP. To assess the association of GGPP with the enzyme, we performed *in vitro* prenylation assays with or without (+)-3-IPEHPC. Then, we analyzed GGPP molecules associated with RGGT by immunoprecipitation of the enzyme using protein-G beads coupled to rabbit anti-RGGT antibody. Excess GGPP was removed by extensive washing of the beads. The RGGT-bound GGPP was then separated from Rab-GG by methanol extraction as Rab-GG precipitates under these conditions. In the absence of inhibitor, RGGT-bound GGPP was isolated when no Rab substrate was added, consistent with the existence of a stable GGPP binding site (Fig. 4). As the yield of enzyme after immunoprecipitation is ~50%, ~80% of RGGT was loaded with radiolabeled GGPP under these conditions (Fig. 4). This RGGT-bound GGPP disappeared in the presence of Rab1a-CC, but not in the presence of Rab1a-CS (Fig. 4). These results suggested that the inability to doubly geranylgeranylate Rab1a-CS led GGPP to remain stuck in the GGPP

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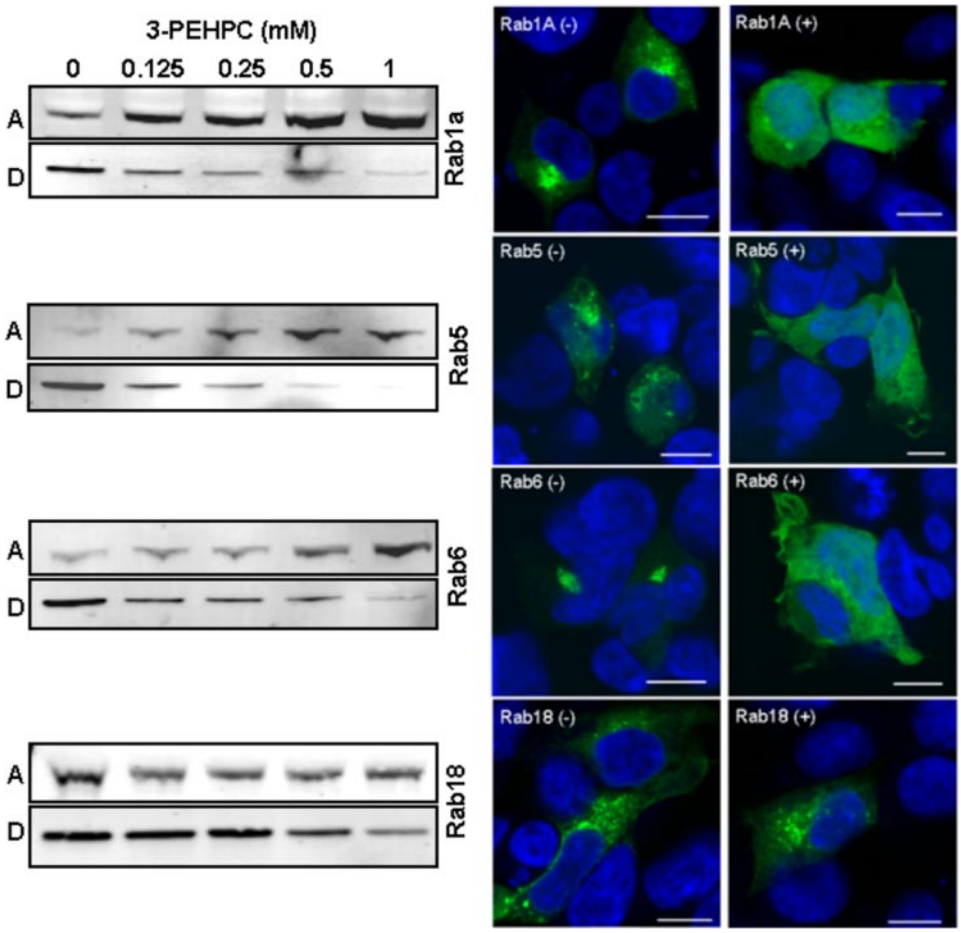


FIGURE 5. **Prenylation and localization of GFP-Rab constructs was analyzed following transfection of 293 cells.** *A*, transfected 293 cells were treated with 0–1 mM 3-PEHPC for 18 h, then prenylated (detergent phase; *D*) and unprenylated proteins (aqueous phase; *A*) were separated by Triton X-114 fractionation and GFP-Rabs detected by Western blotting using an antibody to GFP. *B*, transfected 293 cells were treated with (+) or without (−) 1 mM 3-PEHPC for 18 h, then subcellular localization of GFP-Rabs assessed by confocal microscopy.

binding site. The inhibitor did not compete for the RGGT-bound GGPP in the absence of Rab protein, or in the presence of Rab1a-CS, indicating that it does not preclude the binding of GGPP to RGGT (Fig. 4). These results strongly suggest that the inhibitor binds to a site that is distinct from the GGPP-binding site on RGGT. Interestingly, when inhibitor and Rab1a-CC were both incubated with the enzyme, RGGT-bound GGPP could now be elicited (Fig. 4). This result suggests that the presence of inhibitor with Rab1a-CC leads to freezing of GGPP on the RGGT-binding site, as when Rab1a-CS is present (in the absence of inhibitor). In both cases, the presence of GGPP stuck on RGGT may reflect an inhibition of GG transfer due to an inability of the monoprenylated Rab protein to move to a second site on RGGT, which accepts a newly transferred GG-cysteine.

Prenylation and Localization of Doubly Prenylated Rab Proteins Is Disrupted by PCs in 293 Cells—To assess whether the susceptibility of Rabs with different C-terminal motifs to inhibition by PCs in intact cells matched the *in vitro* assays, we transfected 293 cells with GFP-Rab constructs (Rab1A, Rab5, Rab6, and Rab18) and analyzed the ability of 3-PEHPC to inhibit prenylation *in vivo* and disrupt the localization of these Rab proteins (Fig. 5). Accordingly, we found that prenylation of GFP-Rab1a, GFP-Rab5a, and GFP-Rab6a was susceptible to inhibition by 3-PEHPC, as evidenced by their significant shift from the detergent phase (prenylated forms) to the aqueous phase (unprenylated form) upon Triton X-114 partitioning of cell lysates (Fig. 5*A*). In this experiment, the overexpression leads to the presence of a proportion of GFP-Rab in the aqueous phase even at steady-state. Moreover, 1 mM 3-PEHPC completely prevented the specific membrane targeting of Rab1a, Rab6a, and Rab5a, resulting in a diffuse cytoplasmic localization of these Rabs (Fig. 5*B*), without affecting the growth or viability of the cells over this culture period (data not shown). Reflecting the enzyme inhibition data, the order of potency for inhibition of prenylation by 3-PEHPC was Rab5a > Rab1a > Rab6. In contrast, both the prenylation and subcellular localization of the mono-prenylated, C*AAX* motif-containing Rab18, was resistant to 3-PEHPC treatment, in agreement with the enzyme assays. Furthermore, despite containing a C*AAX* motif, the prenylation and subcellular localization of Rab18 was also unaffected by GGTI-298, a specific inhibitor of GGT-I (data not shown).

Order of Prenyl Addition on Rab Proteins—Two previous studies examined the order of GG addition by RGGT and proposed that the N-terminal cysteine is preferred to the C-terminal one (16, 21). However, the substrates used in those studies were single cysteine mutants of Rab1a and a fluorescent derivative of GGPP, respectively, and not native substrates. Because PCs specifically block only the second event of prenylation, they are useful tools to characterize the first event of Rab prenylation. We took advantage of the fact that the ICMT can only methylate the C-terminal geranylgeranylated cysteine in a C*AAX* or C*XC* motif and not in the context of a CC motif (14). Using a coupled prenylation/methylation assay *in vitro*, we observed as expected, methylation of the Rab1a-SC mutant but not of the Rab1a-CS mutant or wild-type Rab1a-CC (Fig. 6), which is consistent with double prenylation of the CC motif, as previously reported (14). Furthermore, the Rab1a-SC mutant was methylated by ICMT in the presence or absence of inhibitor, confirming the recognition of this motif by the methyltransferase and validating the experimental approach. Note that the inhibitor concentration used in the experiment (100 μM) was below the IC_{50} for Rab1a-CS or SC mutants and therefore would have had little effect on the prenylation reaction. Treatment with (+)-3-IPEHPC did not lead to increased methylation of Rab1a-CC, indicating that pre-

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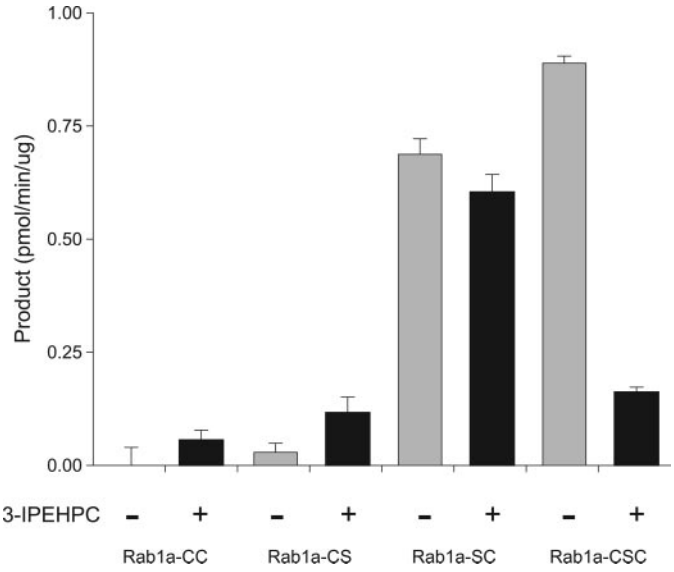


FIGURE 6. **Order of prenyl addition on Rab1a.** The methylation of Rab1a was measured after *in vitro* prenylation of the protein treated with (100 μM) or without (+)-3-IPEHPC (for 30 min at 37 °C). 10 μM [^{3}H]*S*-adenosylmethionine (specific activity = 650 dpm/pmol) and 1 μg of enriched ICMT membranes were added to the reaction mixture for 45 min. Proteins were precipitated and the incorporated radioactivity was measured. This experiment is representative of two other independent experiments.

nylation of the N-terminal cysteine is not affected by the inhibitor. Furthermore, when the Rab1a-CSC mutant was treated with (+)-3-IPEHPC, we observed a dramatic reduction in methylation compared with the untreated reaction, indicating that prenylation of the C-terminal cysteine is inhibited by (+)-3-IPEHPC, *i.e.* this is the second prenylation step. Similar experiments with wild-type Rab27a (CGC) showed identical results (data not shown). These results demonstrate that the N-terminal cysteine in dual-prenylated Rabs is preferred for the first GG addition.

DISCUSSION

We report here a mechanism of action for inhibition of RGGT by PCs. Our data suggests that these inhibitors act as partial inhibitors of the geranylgeranylation reaction, effectively inhibiting only the second GG addition onto those Rab substrates that contain double cysteine motifs at their C terminus. Yet, these behave as effective inhibitors in cell studies presumably for two reasons. One is that the majority of Rabs are doubly geranylgeranylated and the second is that the enzyme exhibits highest affinity for mono-prenylated intermediates in the reaction (16), raising the possibility that these inhibitors sequester the cellular pools of the enzyme in non-productive complexes. Additionally, mono-geranylgeranylation of normally di-geranylgeranylated Rabs, such as Rab5, leads to mistargeting to the endoplasmic reticulum, and loss-of-function (17). Therefore, even if a proportion of inhibitor-induced mono-GG Rabs were able to interact with membranes, they would not be present at the correct cellular location.

A limiting factor to mechanistic studies until now has been the relatively low affinity of 3-PEHPC, the previously reported PC inhibitor of RGGT (10). In this work, we took advantage of a newly synthesized PC derivative, (+)-3-IPEHPC, which we found to be substantially more potent (≥25-fold) than 3-PEHPC at inhibiting RGGT for all the Rab substrates that we tested. The kinetic characterization of RGGT inhibition by these PCs revealed that both compounds are uncompetitive inhibitors with respect to the Rab protein substrate and mixed-type inhibitors with respect to GGPP. We then investigated in more detail the mechanism of inhibition. Using Rab1a C-terminal mutants (CSC, CCS, CS, and SC), we observed that in the presence of the singly prenylated substrates, the efficacy of inhibition was severely impaired. Moreover, the prenylation of Rab proteins with a C*AAX* motif (Rab13, Rab18, and Rab23) was not inhibited by (+)-3-IPEHPC. We hypothesized that only the second event of prenylation is targeted by the PCs and we verified the state of Rab1a and Rab27a prenylation after treatment with PCs. In both cases, the prenylated substrates were modified on a single cysteine, demonstrating that the inhibitors block the second addition of GGPP.

What could be the molecular basis for the inhibition of the second GG transfer? One possibility is that the inhibitors could be interfering with the complex rearrangements that must occur on the active site of RGGT before the prenylation of the second cysteine to remove the first prenyl-cysteine and allow the alignment of that second cysteine with the newly bound second GGPP substrate. Interestingly, Beese and colleagues (22, 23) described several reaction intermediates in the FT and GGT-I reactions that allowed us to create a model for the inhibitory mechanism. These authors showed that soaking FT:prenylated peptide or GGT:prenylated peptide crystals with FPP or GGPP, respectively, led to the movement of the prenylated cysteine from the active site to a new binding site in a solvent accessible groove, instead of dissociation from the enzymes (22, 23). The authors further proposed that a similar process could occur in the RGGT reaction where the first GG-cysteine could be translocated to a nearby second site, allowing the second GG transfer to proceed (22). Our present studies raise the hypothesis that the PC inhibitors bind to this second site for the following reasons. First, experiments measuring the binding of GGPP to RGGT showed that the inhibitors do not preclude GGPP binding and thus suggest that the inhibitors do not bind to the GGPP-binding site. Second, the same experiments showed that the presence of inhibitor froze GGPP in its binding site suggesting that it is inhibiting GGPP transfer by preventing its movement away from the GGPP-binding site. Third, the uncompetitive kinetics *vis à vis* Rab substrates suggest that the inhibitors do not bind to the peptide-binding site.

The mechanism of RGGT inhibition by 3-PEHPC has been studied using a fluorescent analog of GGPP (NBD-FPP) and Rab7a (ending in C*XC*) in a continuous fluorimetric assay (21). Strikingly, the authors observed that the use of NBD-FPP led to the formation of predominantly mono-prenylated Rab7, and not doubly prenylated as observed using the native substrate (24). This inability of NBD-FPP to be used in the second GG addition suggests an obvious reason for the discrepancies between the studies. Furthermore, the present studies suggest that this behavior of NBD-FPP may be related to an inability to bind the putative second site on RGGT. Recently, another study reported on the identification of novel RGGT inhibitors (25). These inhibitors appear to act via a different mechanism, prob-

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ably binding to the peptide site as they exhibit competitive kinetics with respect to Rab and uncompetitive kinetics with respect to GGPP. Nevertheless, all effective inhibitors of this class required a hydrophobic tail, suggesting the possibility that binding to the putative second site may also be a feature of the inhibitory mechanism of these compounds.

Our results imply that the prenylation mechanism for singly and doubly prenylated Rab proteins may be different. Indeed, the prenylation of Rab5a-CVLL is not inhibited by PCs, whereas the prenylation of Rab5a-CCVLL is inhibited. The present data suggests that non-productive ESI complexes do not form in the presence of Rab-C*AAX* substrates, and RGGT is able to dissociate from the mono-GG-Rab after prenylation. In these cases, the *AAX* peptide appears to be of importance because Rab1a-SC or Rab1a-CS are sensitive to inhibition, albeit at high concentrations of the inhibitors (200-fold increase compared with Rab1a WT proteins), whereas Rab-C*AAX* substrates are resistant. One possibility is that the *AAX* tail may prevent the use of the putative second site, which would allow the ready release of the product from the enzyme. Further work will be necessary to understand the molecular basis for these differences.

Finally, we utilized the ability of PCs to inhibit the second event of prenylation to demonstrate that the N-terminal cysteine of doubly prenylated Rabs is preferred for the first event. This is in agreement with our previous report using Rab1a-CS and Rab1a-SC substrates showing that the N-terminal cysteine is more efficiently prenylated than the C-terminal cysteine (16). Moreover, it was reported the first event of Rab7 prenylation was slower than the second one (26) and the prenylation of a Rab7-SXC mutant was 3-fold faster than a Rab7-C*X*S mutant event (21), suggesting the N-terminal cysteine being somewhat preferred for the first prenylation event. We took advantage of the fact that ICMT can only methylate prenylated cysteine when present at the extreme C terminus (14, 27). Here, we observed that Rab1a-CC and Rab1a-CSC substrates in the presence of inhibitor are not methylated by ICMT (Fig. 6), which strongly suggests that the N-terminal cysteine of double cysteine-containing Rabs is preferentially prenylated.

In summary, we have characterized the mechanism of RGGT inhibition by the PC compounds, (+)-3-IPEHPC and 3-PEHPC. These inhibitors do not prevent the substrates binding to RGGT, but block the second event of prenylation possibly by binding to a second prenyl-binding site, to which the first prenyl-cysteine moves into to allow the second GG addition. Crystallization of 3-IPEHPC·RGGT and/or 3-IPEHPC·REP·RGGT·Rab complexes should reveal this putative binding site and the reaction transition states during the complex double geranylgeranylation reaction of Rab proteins. These inhibitors might represent useful novel therapeutic agents in Rab-mediated diseases.

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Enzyme Catalysis and Regulation:

Phosphonocarboxylates Inhibit the Second Geranylgeranyl Addition by Rab Geranylgeranyl Transferase

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