Characterization of human palmitoyl-acyl transferase activity using peptides that mimic distinct palmitoylation motifs

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The covalent attachment of palmitate to proteins commonly occurs on cysteine residues near either N-myristoylated glycine residues or C-terminal farnesylated cysteine residues. It therefore seems likely that multiple palmitoyl-acyl transferase (PAT) activities exist to recognize and modify these distinct palmitoylation motifs. To evaluate this possibility, two synthetic peptides representing these palmitoylation motifs, termed MyrGCK(NBD) and FarnCNRas(NBD), were used to characterize PAT activity under a variety of conditions. The human tumour cell lines MCF-7 and Hep-G2 each demonstrated high levels of PAT activity towards both peptides. In contrast, normal mouse fibroblasts (NIH/3T3 cells) demonstrated PAT activity towards the myristoylated substrate peptide but not the farnesylated peptide, while *ras*-transformed NIH/3T3 cells were able to palmitoylate the FarnCNRas(NBD) peptide. The kinetic parameters for PAT activity were determined using membranes from MCF-7 cells, and indicated that the K_m values for palmitoyl-CoA were identical for PAT activity towards the two substrate peptides; however, the K_m for MyrGCK(NBD) was 5-fold lower than the K_m for FarnCNRas(NBD). PAT activity towards the two substrate peptides was dose-dependently inhibited by 2-bromopalmitate and 3-(1-oxo-hexadecyl)oxiranecarboxamide (16C; IC_{50} values of approx. 4 and 1.3 μM, respectively); however, 2-bromopalmitate was found to be uncompetitive with respect to palmitoyl-CoA, whereas 16C was competitive. To seek additional evidence for multiple PATs, the effects of altering the assay conditions on the palmitoylation of MyrGCK(NBD) and FarnCNRas(NBD) were compared. PAT activity towards the two peptide substrates was modulated similarly by changing the ionic strength or incubation temperature, or by the addition of dithiothreitol. In contrast, the enzymic palmitoylation of the two peptides was differentially affected by *N*-ethylmaleimide and thermal denaturation. Overall, these data demonstrate that the enzymic palmitoylation of farnesyl- and myristoyl-containing peptide substrates can be differentiated, suggesting that multiple motif-specific PATs exist.

Key words: 2-bromopalmitate, palmitoylation, palmitoyl-acyl transferase, palmitoyl-coenzyme A, 3-(1-oxo-hexadecyl)oxirane-carboxamide (16C).

INTRODUCTION

Protein palmitoylation is an important post-translational lipid modification in which a 16-carbon palmitate group is attached to a cysteine residue of specific proteins via a thioester bond [1]. Similar to other lipid modifications, such as myristoylation and isoprenylation, palmitoylation increases the hydrophobicity of a protein and promotes association of these proteins with specific membranes [1–6]. Palmitoylation facilitates the localization of proteins to the plasma membrane, and more particularly to microdomains called lipid rafts or caveolae within the plasma membrane [7,8]. Compared with other stable lipid modifications, palmitoylation is a reversible lipid modification under the control of two types of enzyme [9,10]: (i) palmitoyl-acyl transferases (PATs) that catalyse the attachment of palmitate to proteins and (ii) thioesterases that cleave the palmitate from proteins [11–13]. Although palmitate cleavage has been well characterized through the identification, purification and cloning of several thioesterases, the mechanism and enzymic properties of the palmitoylation reaction remain relatively unstudied, due to the lack of success in purifying and cloning PATs.

Palmitoylation is an important modification for localizing several proteins to the plasma membrane, including proteins that play critical roles in signal-transduction pathways. Although the proteins that are modified by palmitoylation are divergent in function, similarities between the sites of palmitoylation allow them to be sorted into four groups [14]. One group of proteins includes transmembrane, integral or peripheral membrane proteins, such as transforming growth factor α, serotonin receptors, dopamine D1 receptors and HIV-1 gp160, that are palmitoylated on cysteine residues located in close proximity to transmembrane domains. A second group of proteins, including certain Gα subunits, GAP43 and PSD-95, is palmitoylated near the N- or C-terminus. These first two groups of proteins do not contain an overt consensus sequence for palmitoylation and do not require prior lipidation for palmitoylation to occur. A third group of proteins, including H-Ras, N-Ras and paralemmin, is first farnesylated at a C-terminal CAAX box and then modified further with palmitate at a nearby cysteine residue [15]. Finally, a fourth group of proteins, including endothelial nitric oxide synthase, certain Src-family tyrosine kinases and certain Gα subunits, are first myristoylated at an N-terminal glycine residue and then palmitoylated at a nearby cysteine residue [6].

The mechanism for protein palmitoylation is currently under debate, with evidence supporting both enzymic [16–19] and non-enzymic [20–22] palmitoylation. It is likely that the mechanism depends on the substrate protein. Of the four

Abbreviations used: IVP, *in vitro* palmitoylation; PAT, palmitoyl-acyl transferase; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; 16C, 3-(1-oxo-hexadecyl)-oxiranecarboxamide; 2-BP, 2-bromopalmitate; NP-40, Nonidet P40; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; NBD C_{12}-HPC, 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecaboyl-*sn*-glycero-3-phosphocholine.

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groups of palmitoylated proteins discussed above, it is likely that palmitoylation of proteins in groups 1 and 2 involves non-enzymic modification of an exposed cysteine residue. In contrast, proteins in groups 3 and 4 have distinct recognizable palmitoylation motifs at their C- and N-terminal regions, suggesting enzymic palmitoylation of these substrates. Further, since the palmitoylation motifs of these two groups of proteins are clearly distinct, it is possible that different isozymes of PAT exist to recognize and modify these motifs. This is supported by recent reports indicating that the yeast *Saccharomyces cerevisiae* has at least two PAT enzymes that recognize distinct protein substrates [23,24].

In spite of the importance of protein palmitoylation in regulating cell function, little is known about the properties, including the multiplicity, of PAT. Attempts to purify and biochemically characterize human PAT enzymes have had only limited success due to the instability of the activity during purification. Small peptides mimicking acylated protein substrates have been useful tools for the characterization of other lipid-modifying enzymes, such as farnesyltransferases and N-myristoyltransferases [25–31], and may also be useful for characterization of PAT enzymes. Recently we have described a sensitive HPLC-based *in vitro* palmitoylation (IVP) assay system that demonstrated clearly that membranes from human tumour cells express PAT activity towards a peptide substrate that mimics the N-terminal myristoyl palmitoylation motif [18]. In the present studies, we have extended the IVP assay to include a peptide substrate that mimics the C-terminal farnesyl palmitoylation motif, and demonstrate that enzymic activity towards these two substrates can be differentiated.

MATERIALS AND METHODS

Materials

The MyrGCK(NBD) peptide substrate (Figure 1) was synthesized by Dr Blake R. Peterson at the Pennsylvania State University (University Park, PA, U.S.A.) as described previously [32]. The FarnCNRas(NBD) peptide substrate (Figure 1) was synthesized in our laboratory as described previously [33]. Palmitoyl-CoA, *N*-ethylmaleimide (NEM), dithiothreitol (DTT), Nonidet P40 (NP-40), NaCl and glutathione were from Sigma (St. Louis, MO, U.S.A.). 2-Bromopalmitate (2-BP), also known as 2-bromohexadecanoic acid, was purchased from Aldrich (Milwaukee, WI, U.S.A.). 3-(1-Oxo-hexadecyl)oxiranecarboxamide (termed 16C) was synthesized in our lab as described previously [34]. *O*-t-Butyl-L-serine methyl ester [H-Ser(tBu)-OMe] was from Novabiochem (San Diego, CA, U.S.A.). The C_4-HPLC column was from Fisher Scientific (Fair Lawn, NJ, U.S.A.) and the 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecaboyl-*sn*-glycero-3-phosphocholine (NBD C_{12}-HPC) internal standard was from Molecular Probes (Eugene, OR, U.S.A.).

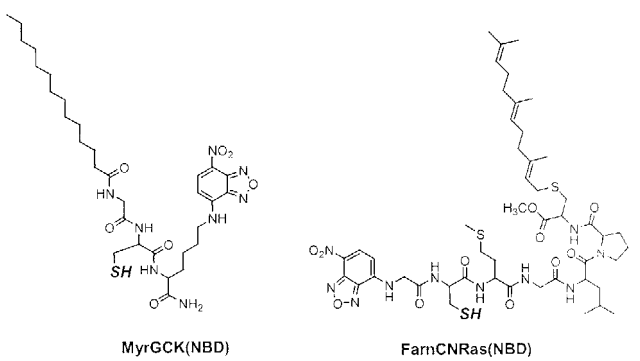

Figure 1 Structures of the two peptides used as substrates in the IVP assays

The MyrGCK(NBD) peptide is a three-amino-acid peptide that mimics the myristoylated N-terminus of $p59^{fyn}$, and has the sequence N-myr-Gly-Cys-Lys-NBD. The FarnCNRas(NBD) peptide is a seven-amino-acid peptide that mimics the farnesylated C-terminus of N-Ras, with the sequence of NBD-Gly-Cys-Met-Gly-Leu-Pro-Cys(farn)-*O*-methyl. The site of palmitoylation on each peptide is indicated as the emboldened and italicized thiol group.

Cell culture

Hep-G2 cells (A.T.C.C. HB-8065) were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 50 μg/ml gentamycin and 1 mM sodium pyruvate at 37 °C in an atmosphere of 5% CO_2/95% air. MCF-7 cells (A.T.C.C. HTB-22) were maintained in RPMI 1640 medium containing 10% fetal bovine serum and 50 μg/ml gentamycin. NIH/3T3 wild-type (A.T.C.C. CRL-1658), EJ-transformed, N-Ras-transformed and K-Ras-transformed cell lines were all maintained in Dulbecco's modified Eagle's medium containing 10% calf serum, 50 μg/ml gentamycin and 1 mM sodium pyruvate.

Preparation of subcellular fractions

To determine the optimal subcellular fraction for use in the IVP assay, cells were fractioned as described in Smith et al. [35]. Briefly, cells were grown to 70% confluence in 150-mm tissue-culture dishes and collected by centrifugation at 600 ***g*** for 10 min at 4 °C. The cells were swollen with hypotonic lysis buffer containing 10 mM Hepes (pH 7.4), 10 mM KCl, 1.5 mM $MgCl_2$ and 5 μM PMSF for 30 min on ice. The cells were disrupted by homogenization and centrifuged at 5600 ***g*** for 10 min at 4 °C to remove nuclei and debris. The supernatant from the low-speed centrifugation was then centrifuged at 100 000 ***g*** for 1 h at 4 °C. The resulting pellet from this ultracentrifugation was resuspended in lysis buffer and collected as the membrane fraction for use in the IVP assays. The membrane fraction was assayed for protein concentration with a fluorescamine assay [36] and used immediately following this preparation.

IVP assay method

The MyrGCK(NBD) peptide substrate was stored as the t-butyl-disulphide precursor and was deprotected immediately before use by incubation (peptide concentration, 10 mM) with β-mercaptoethanol/DMSO (28:72, v/v) containing 17 mM Tris base at 55 °C for 15 min with agitation. The FarnCNRas(NBD) substrate was incubated similarly with β-mercaptoethanol/DMSO (28:72, v/v) containing 17 mM Tris base at 55 °C for 15 min with agitation immediately before use in the IVP assay to cleave any disulphide bonds formed during storage at −80 °C. Unless otherwise noted, IVP assays were performed as described previously [18]. Briefly, IVP assays with the MyrGCK(NBD) substrate consisted of 10 μM deprotected MyrGCK(NBD) peptide, 2 μM palmitoyl-CoA, 50 μg of membrane protein and acylation buffer (50 mM citrate, 50 mM phosphate, 50 mM Tris and 50 mM 3-(cyclohexylamino)propane-1-sulphonic acid ('Caps'), pH 7.2) in a total volume of 100 μl. IVP assays with the FarnCNRas(NBD) peptide consisted of the same IVP assay mixture as above except with optimized concentrations of 20 μM peptide and 4 μM palmitoyl-CoA. The IVP assay was performed by incubating the peptide substrate with the membranes in acylation buffer for 8 min at 37 °C with agitation.

Palmitoyl-CoA was then added to the mixture to start the reaction and incubation was continued at 37 °C for an additional 7.5 min. The assay was stopped upon addition of 1.2 ml of dichloromethane/methanol/water (2:1:1, by vol.), which also extracts the peptide into the organic phase. Phases were separated by low-speed centrifugation and the organic phase was removed to another tube. Additional dichloromethane was added to the aqueous phase to repeat the extraction twice. The dichloromethane fractions were combined, dried under N_2 and stored at −20 °C until they were analysed as indicated below.

HPLC method

The HPLC unit consisted of the Beckman Coulter System Gold and a Waters 470 scanning fluorescence detector. The assay extracts were dissolved in 27 μl of DMSO and peptides were resolved on a reversed-phase wide-pore butyl column (5 μM; 300 Å; 4.6 mm × 250 mm) using an acetonitrile gradient with a flow rate of 1 ml/min. Initially, the mobile phase was maintained as 50 % water/50 % acetonitrile/0.1 % trifluoroacetic acid for 5 min, followed by a 5 min linear gradient of 50–100 % acetonitrile. The mobile phase was then maintained at 100 % acetonitrile for 8 min, followed by a linear gradient from 100 % to 50 % acetonitrile over 2 min. The 7-nitrobenz-2-oxa-1,3-diazol-4-yl (NBD)-labelled peptides were detected by their fluorescence at their optimal excitation and emission wavelengths of 465 and 531 nm, respectively. The limit of detection was approx. 4.7 pmol of NBD-labelled peptide, as determined previously with NBD C_{12}-HPC used as an internal standard. Based on the ratio of palmitoylated to native peptide measured, i.e. peak C/peak A in Figure 2 (see below), the mass of peptide converted into the palmitoylated form could be determined. Because there is a low rate of chemical palmitoylation of the peptides, it was necessary to determine this rate of autoacylation under each of the assay conditions described in the Results section. Enzymic palmitoylation was calculated by subtracting the level of autoacylation from the level of total palmitoylated peptide in each assay. Statistical analyses were performed with InStat data-analysis programs using one-way ANOVA. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

Resolution of the FarnCNRas(NBD) peptides by HPLC

We have demonstrated previously that the peptide MyrGCK(NBD) (Figure 1) can be palmitoylated enzymically *in vitro* by incubation with membrane fractions from MCF-7 or Hep-G2 cells [18]. We now extend those studies using the synthetic FarnCNRas(NBD) that mimics the C-terminus of N-Ras and has the sequence NBD-Gly-Cys-Met-Gly-Leu-Pro-Cys(farn)-*O*-methyl (Figure 1). This peptide contains an N-terminal NBD fluorescent tag, a C-terminal farnesylated and methylated cysteine residue, and a palmitoylation site at a remaining cysteine residue. Palmitoylation of these peptides results in significant changes in the hydrophobicities of the peptides, so that the native and palmitoylated peptides can be resolved by reversed-phase HPLC and quantified using fluorescence detection of the NBD tags. This was demonstrated previously for the MyrGCK(NBD) peptide [18], and resolution of the native and palmitoylated form of the FarnCNRas(NBD) peptide is shown in Figure 2. Incubation of 20 μM FarnCNRas(NBD) at 37 °C in modified acylation buffer (pH 8.2) alone for 15 min resulted in two peaks of fluorescence with retention times of 12.4 and 14.0 min (Figure 2, peaks A and B, respectively). Peak A represents the native farnesylated

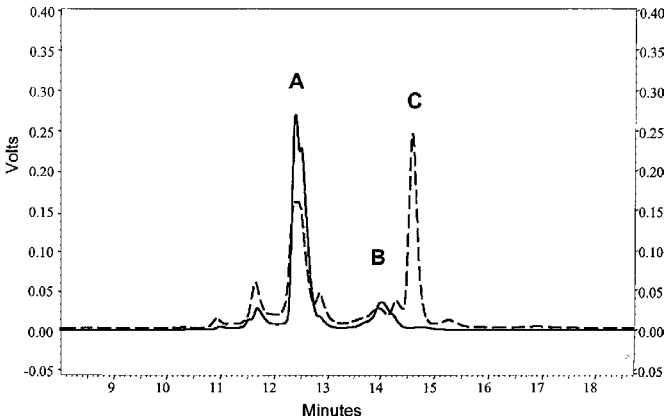

Figure 2 Chromatograms of the FarnCNRas(NBD) peptide and the palmitoylated FarnCNRas(NBD) peptide

The solid trace represents 20 μM peptide incubated at 37 °C in modified acylation buffer (pH 8.2) for 15 min in the absence of palmitoyl-CoA. The dashed trace represents the chromatogram of autoacylation of 20 μM peptide with 200 μM palmitoyl-CoA for 15 min at 37 °C in modified acylation buffer (pH 8.2). The peptide was extracted out of the reaction mix as described in the Materials and methods section. Peak A, FarnCNRas(NBD); peak B, disulphide-linked FarnCNRas(NBD); peak C, palmitate-modified FarnCNRas(NBD).

peptide, whereas peak B represents a disulphide-linked peptide dimer. Chemical palmitoylation of FarnCNRas(NBD) was accomplished by incubating the peptide with a high concentration of palmitoyl-CoA at pH 8.2, approximately the pK_a of the cysteine residue, thereby creating a thiolate anion to drive the autoacylation of the peptide [21]. After incubating 20 μM FarnCNRas(NBD) with 200 μM palmitoyl-CoA at 37 °C at pH 8.2 for 15 min, a new peak was eluted with a retention time of 14.6 min (Figure 2, peak C). As expected under these chemical palmitoylating conditions, and similar to results with the MyrGCK(NBD) peptide [18], peak C represented the palmitate-modified form of the FarnCNRas(NBD) peptide. Analysis of peak C by high-resolution MS with positive-ion electrospray ionization (performed by The Scripps Research Institute, La Jolla, CA, U.S.A.) verified that it was the palmitoylated form of the FarnCNRas(NBD) peptide, with an expected mass of 1300 Da (results not shown).

Enzymic palmitoylation of the MyrGCK(NBD) and FarnCNRas(NBD) peptides

Membranes isolated from a panel of cell lines were tested for their ability to enzymically palmitoylate the two PAT substrate peptides. In contrast to the chemical palmitoylation assays described above, enzymic IVP assays were performed at a physiological pH (pH 7.2) as this results in low rates of peptide autoacylation. Typically, the autoacylation rates were between 4 and 10 % of those measured in the presence of PAT. These background autoacylation levels were subtracted from total palmitoylation levels in the IVP assays, so that only enzymic PAT activities towards the peptide substrates are reported in all of the following data. As shown in Figure 3, the FarnCNRas(NBD) peptide substrate was efficiently palmitoylated when incubated with membrane fractions from the human tumour cell lines MCF-7 or Hep-G2. The FarnCNRas(NBD) substrate was also enzymically palmitoylated by membranes isolated from NIH/3T3 cells transformed by either N-Ras, K-Ras or H- (EJ-)Ras, whereas the untransformed parental NIH/3T3 cells did not express PAT activity towards FarnCNRas(NBD). The MyrGCK(NBD) peptide substrate was also efficiently palmitoylated when incubated with

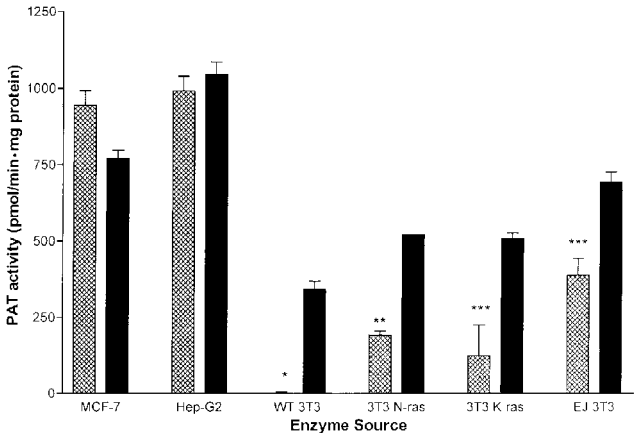

Figure 3 *In vitro* enzymic palmitoylation of the FarnCNRas(NBD) or MyrGCK(NBD) peptide using membranes from various cell lines

FarnCNRas(NBD) peptide (cross-hatched bars) or MyrGCK(NBD) peptide (black bars) was incubated with membrane fractions from the indicated cell lines for 8 min and then incubated with palmitoyl-CoA for an additional 7.5 min at 37 °C before analysis as described in the Materials and methods section. Values represent means ± S.D. of PAT activity. Results are from $n = 30$, 21, 6, 6, 6 or 13 assays with membranes from MCF-7 cells, Hep-G2 cells, wild-type NIH/3T3 cells (WT 3T3), or 3T3 cells transfected with oncogenic N-*ras*, K-*ras* or EJ-*ras*, respectively. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P = 0.001$ for differences in PAT activity between the FarnCNRas(NBD) and MyrGCK(NBD) substrates within the cell line indicated.

membrane fractions from MCF-7, Hep-G2 or *ras*-transformed 3T3 cells. In contrast with the FarnCNRas(NBD) substrate, MyrGCK(NBD) was palmitoylated by the membrane fraction from wild-type NIH/3T3 cells, suggesting that different PAT activities may be expressed upon transformation of the cells by *ras* oncogenes.

Membranes from MCF-7 cells displayed the highest and most consistent PAT activity towards both peptide substrates, and so were used as the primary model for subsequent experiments described below. However, we have verified a number of critical experiments using membranes from Hep-G2 cells, and these results consistently paralleled the data from experiments using MCF-7 membranes. First, time-course experiments demonstrated that the enzymic palmitoylation of both the MyrGCK(NBD) and FarnCNRas(NBD) peptides was linear with time until approx. 75% of the native peptide was depleted (results not shown). The standard time for subsequent IVP assays was set at 7.5 min to remain in the linear region of the assay. Next, the Michaelis–Menten kinetic parameters for the palmitoyl-CoA and peptide substrates were determined. Because the rate of peptide autoacylation is also affected by the concentration of palmitoyl-CoA, the concentration range of palmitoyl-CoA was chosen to minimize chemical palmitoylation while still giving measurable enzymic palmitoylation. As indicated in Figure 4(A), titration of palmitoyl-CoA from 1 to 8 μM for palmitoylation of the MyrGCK(NBD) peptide gave a parabolic curve with a V_{max} of 32 ± 2 pmol/min and a K_m of 0.95 μM for palmitoyl-CoA ($R^2 = 0.989$; Table 1). For the FarnCNRas(NBD) peptide (Figure 4B), titration of palmitoyl-CoA from 2 to 16 μM also fitted a parabolic curve with a V_{max} of 67 ± 2 pmol/min and a K_m of 0.79 μM for palmitoyl-CoA ($R^2 = 0.994$; Table 1). The concentration dependence for each peptide in the PAT assay was also examined. Titration of the MyrGCK(NBD) peptide from 10 to 80 μM gave a parabolic curve with a V_{max} of 17.4 ± 0.2 pmol/min and a K_m of 1.5 ± 0.2 μM ($R^2 = 0.999$; Table 1). For the FarnCNRas(NBD) peptide, titration from 20 to 160 μM also fitted a parabolic curve with a V_{max} of 35 ± 1 pmol/min with

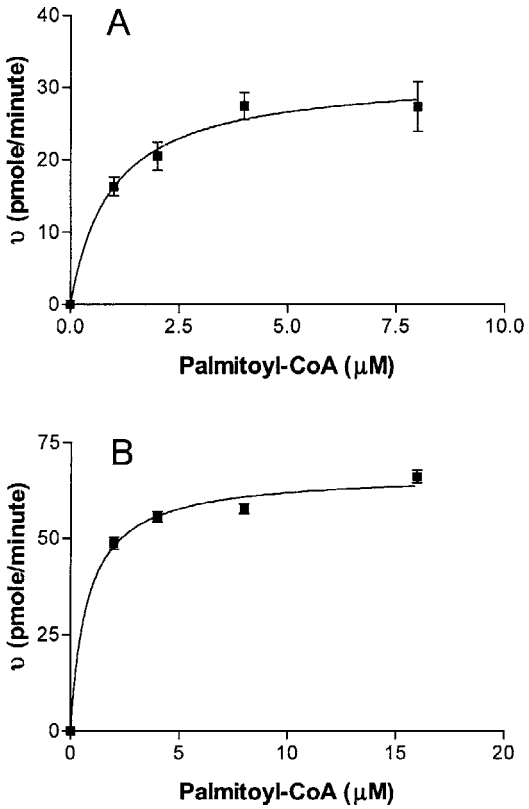

Figure 4 Characterization of the concentration dependence of palmitoyl-CoA on palmitoylation of the FarnCNRas(NBD) and MyrGCK(NBD) substrates

(**A**) MyrGCK(NBD) peptide was incubated with membrane fractions from MCF-7 cells (50 μg of protein) for 8 min and then incubated with 1, 2, 4 or 8 μM palmitoyl-CoA for an additional 7.5 min at 37 °C before analysis as described in the Materials and methods section. (**B**) FarnCNRas(NBD) peptide was incubated with membrane fractions from MCF-7 cells (50 μg of protein) for 8 min and then incubated with 2, 4, 8 or 16 μM palmitoyl-CoA for an additional 7.5 min at 37 °C before analysis as described in the Materials and methods section. Values are means ± S.D. of PAT activity from three independent experiments. Fit parameters for both plots are given in Table 1.

Table 1 Kinetic parameters for palmitoyl-CoA and peptide titrations

Substrate	Parameter	FarnCNRas(NBD)	MyrGCK(NBD)
Palmitoyl-CoA	V_{max} (pmol/min)	67 ± 2	32 ± 2
	K_m (μM)	0.79 ± 0.19	0.95 ± 0.21
	Goodness of fit (R^2)	0.994	0.99
Peptide	V_{max} (pmol/min)	35 ± 1	17 ± 0
	K_m (μM)	7.8 ± 1.2	1.5 ± 0.2
	Goodness of fit (R^2)	0.998	1.00

a K_m of 7.8 ± 1.2 μM ($R^2 = 0.997$; Table 1). Evaluation of the kinetics of both the lipid substrate and the peptide substrates of the PAT assay demonstrated clear concentration dependences and saturation, supporting the hypothesis that these reactions represent enzymic processes.

Characterization of PAT activity towards MyrGCK(NBD) and FarnCNRas(NBD) peptide substrates

Because a variety of cellular proteins undergo palmitoylation, it is attractive to hypothesize that multiple PAT enzymes exist to palmitoylate distinct motifs in these protein substrates. We used the MyrGCK(NBD) and FarnCNRas(NBD) peptides to

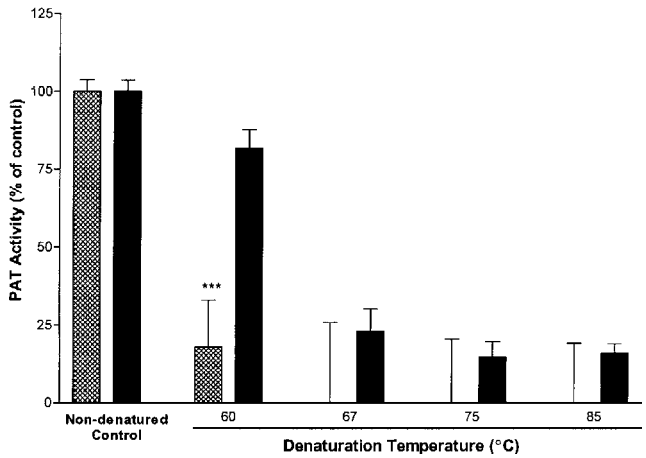

Figure 5 Effects of thermal denaturation on PAT activity towards the FarnCNRas(NBD) and MyrGCK(NBD) substrates

IVP assays were performed with MCF-7 membranes that had been incubated at 60, 67, 75 or 85 °C for 10 min prior to the assay. Values represent the means ± S.D. of PAT activity towards FarnCNRas(NBD) (cross-hatched bars) or MyrGCK(NBD) (black bars). Results from six assays are shown. ***$P < 0.001$ compared with PAT activity towards MyrGCK(NBD) under the same treatment.

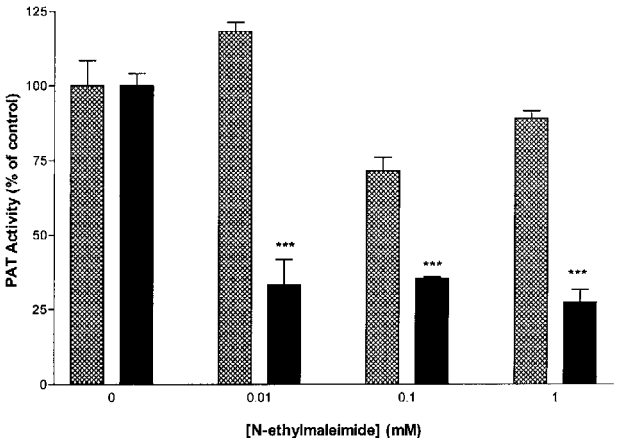

Figure 6 Effects of NEM on PAT activity towards the FarnCNRas(NBD) and MyrGCK(NBD) substrates

IVP assays were performed with MCF-7 membranes that had been treated with 0, 0.01, 0.1 or 1 mM NEM for 30 min at 37 °C followed by treatment with 0.02, 0.2 or 2 mM reduced glutathione, respectively, for 10 min at 37 °C prior to the assay. Values represent means ± S.D. of PAT activity towards FarnCNRas(NBD) (cross-hatched bars) or MyrGCK(NBD) (black bars). Results from three assays are shown. ***$P < 0.001$ compared with PAT activity towards FarnCNRas(NBD) at the same NEM concentration.

compare PAT activity towards the C-terminal farnesyl motif and the N-terminal myristoyl motif under various biochemical and pharmacological assay conditions. Palmitoylation of both FarnCNRas(NBD) and MyrGCK(NBD) was relatively insensitive to ionic strength changes up to 250 mM NaCl, but 500 mM NaCl reduced the PAT activity towards both peptides by approx. 50 %. Similarly, varying the assay incubation temperature from 25 to 42 °C had only modest effects on PAT activity; however, non-enzymic palmitoylation of both peptides was increased at 42 °C, making 37 °C the optimal assay temperature. Evaluating the response to increasing detergent concentrations, however, revealed differences in PAT activity towards the FarnCNRas(NBD) and MyrGCK(NBD) substrates. Using either MCF-7 or Hep-G2 membranes, PAT activity towards MyrGCK(NBD) was inhibited strongly by the presence of the detergent NP-40. In contrast, NP-40 at concentrations up to at least 1 % did not affect PAT activity towards the FarnCNRas(NBD) substrate ($P < 0.05$ and $P < 0.001$ for differences between the peptides at 0.01 and 1 % NP-40, respectively).

To examine the thermal stability of PAT activity, membranes from MCF-7 cells were incubated at 60, 67, 75 or 85 °C before the IVP assay was conducted at the normal temperature of 37 °C. PAT activity towards the MyrGCK(NBD) peptide substrate significantly decreased by 18, 77, 85 and 84 % after incubation at 60, 67, 75 and 85 °C, respectively (Figure 5). In comparison, PAT activity towards the FarnCNRas(NBD) peptide substrate was more sensitive to thermal denaturation, being inhibited by 82 % after incubation at 60 °C and by 100 % at all higher temperatures. Although PAT activity towards the FarnCNRas(NBD) peptide was more sensitive to denaturation than was PAT activity towards the MyrGCK(NBD) peptide at all temperatures, this difference was most significant at 60 °C ($P < 0.001$). Similar thermal-sensitivity studies were performed with membranes from Hep-G2 cells, with similar results (not shown).

A likely mechanism for PAT activity involves the transfer of the palmitoyl moiety from palmitoyl-CoA to a cysteine residue on PAT, followed by its transfer to the substrate protein. To investigate this possibility, the effects of the thiol-reducing agent DTT and the thiol-alkylating agent NEM [11,37] on PAT activity towards the FarnCNRas(NBD) and MyrGCK(NBD) peptide substrates were examined (Figure 6). As NEM covalently alkylates any thiol moiety it must be removed from the reaction prior to introduction of the peptide substrates in the IVP assay. Therefore, a 2-fold excess of reduced glutathione was added to the reaction prior to the addition of peptide substrates to effectively sequester any free NEM remaining in the reaction. Treatment of MCF-7 membranes with DTT concentrations of 0.5 mM or lower did not affect PAT activity towards either peptide substrate. In contrast, 5 mM DTT inhibited the activity towards both substrates by approx. 30 %. This may reflect cleavage of the palmitoyl intermediate from PAT before it can be transferred to the peptide substrate. PAT activity towards the FarnCNRas(NBD) peptide was only slightly inhibited by treatment of MCF-7 membranes with up to at least 1 mM NEM (Figure 6). In contrast, PAT activity towards the MyrGCK(NBD) peptide was inhibited by 65–73 % by NEM (Figure 6). Significant differences were observed in PAT activity towards the MyrGCK(NBD) and FarnCNRas(NBD) substrates after treatment with 0.01, 0.1 and 1 mM NEM ($P < 0.001$).

Effects of pharmacological agents on PAT activity towards the MyrGCK(NBD) and FarnCNRas(NBD) peptide substrates

To characterize differential PAT activities further, we examined the effects of various palmitoylation inhibitors in the IVP assay using the MyrGCK(NBD) and FarnCNRas(NBD) peptide substrates. One known palmitoylation inhibitor is 2-BP, which has been shown to effectively inhibit palmitoylation in intact cells [38]. When MCF-7 membranes were treated with increasing concentrations of 2-BP, PAT activity towards both the MyrGCK(NBD) and FarnCNRas(NBD) peptide substrates was dose-dependently inhibited (Figure 7A). Inhibition of PAT activity towards MyrGCK(NBD) and FarnCNRas(NBD) by 2-BP had nearly identical IC_{50} values of approx. 4 μM. We also studied the effects of a second palmitoylation inhibitor, termed 16C, which was designed to mimic palmitoyl-CoA and was shown previously to be an effective inhibitor of Ras palmitoylation in intact cells [34]. As shown in Figure 7(B), 16C also dose-dependently inhibited PAT activity towards both peptide substrates, reaching

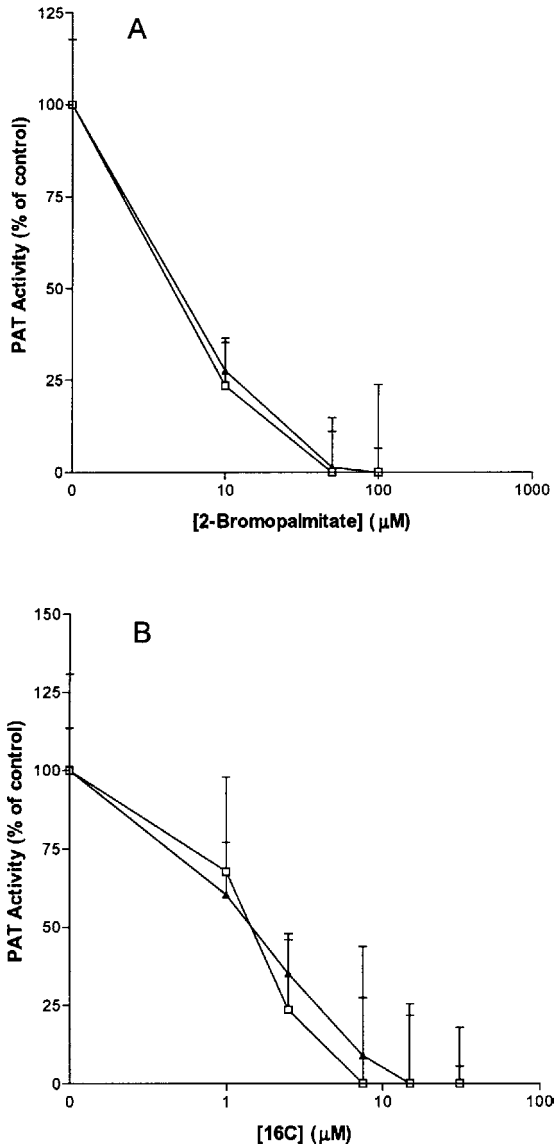

Figure 7 Effects of 2-BP and 16C on PAT activity towards the FarnCNRas(NBD) and MyrGCK(NBD) substrates

IVP assays were performed with MCF-7 membranes treated with the indicated concentrations of (**A**) 2-BP or (**B**) 16C for 10 min at 37 °C prior to the assay. Values represent means ± S.D. of PAT activity towards FarnCNRas(NBD) (□) or MyrGCK(NBD) (▲). Results from six (**A**) and eight (**B**) assays are shown.

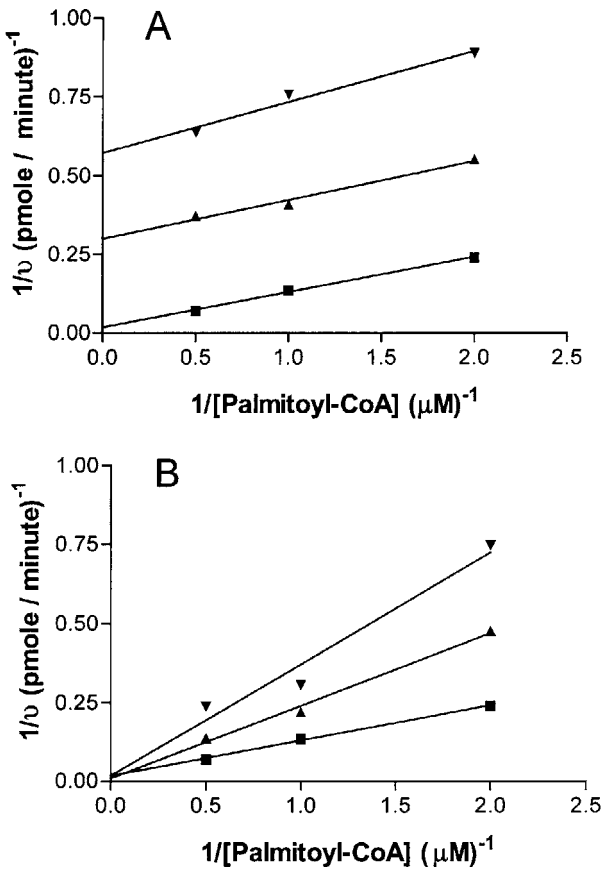

Figure 8 Analysis of the mechanism of inhibition of palmitoylation by 2-BP and 16C

Membrane fractions from MCF-7 cells (50 μg of protein) were incubated with either 2-BP or 16C for 15 min at 37 °C. MyrGCK(NBD) peptide was added to the reaction and incubated for 8 min at 37 °C followed by the addition of palmitoyl-CoA and a final incubation for 7.5 min at 37 °C before analysis as described in the Materials and methods section. (**A**) Double-reciprocal plots of 0.5, 1 or 2 μM palmitoyl-CoA in the presence of 0 (■), 4.4 (▲) or 10 (▼) μM 2-BP. (**B**) Double-reciprocal plots of 0.5, 1 or 2 μM palmitoyl-CoA in the presence of 0 (■), 1.4 (▲) or 5 (▼) μM 16C. Values represent the mean of PAT activity from three independent experiments. Fit parameters for both plots are given in Table 2.

Table 2 Parameters for Lineweaver–Burk plots (Figure 8)

Compound	Concentration (μM)	Slope	*y* Intercept	*x* Intercept	R^2
2-BP	0	0.112 ± 0.007	0.018 ± 0.009	−0.16	0.996
	4.4	0.124 ± 0.017	0.30 ± 0.022	−2.42	0.981
	10	0.161 ± 0.027	0.573 ± 0.036	−3.56	0.973
16C	0	0.112 ± 0.007	0.018 ± 0.009	−0.16	0.996
	1.4	0.231 ± 0.022	0.009 ± 0.03	−0.04	0.991
	5	0.353 ± 0.075	0.018 ± 0.099	−0.05	0.957

complete inhibition by 7.5 μM. Again, nearly identical IC_{50} values of approx. 1.3 μM were observed for the MyrGCK(NBD) and FarnCNRas(NBD) peptides. Assays to study the effects of 2-BP and 16C on PAT activity in Hep-G2 membranes yielded similar results (not shown).

To further examine the mechanisms of inhibition of PAT by the lipid analogues 2-BP and 16C, competition studies with palmitoyl-CoA were conducted using the MyrGCK(NBD) substrate. Double-reciprocal plots of palmitoyl-CoA titrations from 0.5 to 2 μM in the presence of 0, 4.4 or 10 μM 2-BP generated nearly parallel lines (Figure 8A; see Table 2 for fit parameters), indicating that 2-BP acts as a non-competitive inhibitor with respect to palmitoyl-CoA. Similar kinetic analysis of the mechanism of 16C inhibition showed that it is a competitive inhibitor of the palmitoylation reaction with respect to palmitoyl-CoA (Figure 8B). Double-reciprocal plots of palmitoyl-CoA titrations from 0.5 to 2 μM in the presence of 0, 1.4 or 5 μM 16C generated lines that converged at the *y* axis (see Table 2 for fit parameters). A plot of the apparent K_m versus the concentration of 16C indicated a K_i of 2.55 μM.

DISCUSSION

As described earlier, proteins that are enzymically palmitoylated often contain a putative targeting motif consisting of either a farnesylated cysteine residue at the C-terminus or a myristoylated

glycine at the N-terminus. It therefore seems likely that multiple PAT enzymes exist to palmitoylate protein substrates containing these specific motifs. Previously we described a highly sensitive IVP assay that allows the analysis of enzymic palmitoylation of fluorescently labelled peptide substrates by HPLC [18]. With this IVP assay system, we are able to distinguish autoacylation from enzymic palmitoylation of the peptide substrate, so that properties of PAT can be characterized. The IVP assay system was developed using the peptide MyrGCK(NBD), which mimics proteins with an N-terminal myristoylated glycine followed by a palmitoylatable cysteine residue [32]. Using this peptide, PAT activity was identified in cellular membrane fractions from tumour cells. To extend these studies, we sought to develop a similar IVP assay system to evaluate enzymic palmitoylation with a peptide substrate containing the other major palmitoylation motif. Therefore, we synthesized the peptide substrate FarnCNRas(NBD) that mimics proteins with a C-terminal farnesylated cysteine and an upstream palmitoylatable cysteine, e.g. N-Ras [33]. Studies described herein demonstrate that the peptide is also suitable for studies of its chemical and enzymic palmitoylation using the HPLC separation method. Kinetic studies have demonstrated that the palmitoylation of each of the peptides is time-dependent, temperature-sensitive and saturable by both palmitoyl-CoA and the peptide substrate. These data strongly support the hypothesis that PAT enzymes mediate the palmitoylation of these peptides.

The highly sensitive IVP assay also allowed us to characterize PAT activity with respect to palmitoyl-CoA binding. The compounds 16C and 2-BP were shown to act as inhibitors of PAT, albeit with different biochemical mechanisms. As hypothesized in its design, 16C was found to act as a competitive inhibitor with respect to palmitoyl-CoA. In contrast, the finding that 2-BP acts as a non-competitive inhibitor was a somewhat surprising. However, Coleman et al. [39] have found that 2-BP inhibits mono- and di-acylglycerol acyltransferases in a manner other than competing for the acyl-CoA-binding site. The potencies of 16C and 2-BP for inhibition of PAT activity towards both peptide substrates were nearly identical under all conditions, indicating a low level of probability for differentially inhibiting PATs using lipid substrate analogues.

Studying the mechanism of protein palmitoylation is necessary for understanding how this modification affects the function of various palmitoylated proteins. It is known that palmitoylation is important for anchoring proteins in the plasma membrane. However, recent evidence has demonstrated a potential role for palmitoylation in the translocation of various proteins from intracellular compartments, such as the endoplasmic reticulum, to the plasma membrane. For example, palmitoylation of the Gα subunit of certain heterotrimeric G-proteins has been shown to be required for translocation of the G$\alpha\beta\gamma$ heterotrimeric complex to the plasma membrane [40]. In addition, inhibition of palmitoylation of various RhoGTPases, such as RhoB and TC10, results in mislocalization of these proteins to the endoplasmic reticulum, demonstrating a requirement for palmitoylation of these proteins for plasma-membrane targeting [41]. Other proteins for which palmitoylation has been shown to be a requirement for transport from intracellular compartments to the plasma membrane include caveolin-1 [42], CCR5 [43] and H-Ras [44]. From these studies, it appears that palmitoylation plays important roles in the anchorage of proteins in the plasma membrane and in the transport of proteins to the plasma membrane.

In addition to being a targeting signal, palmitoylation may also influence the function of the protein that is palmitate-modified. For example, proteins such as the β2-adrenergic receptor are palmitoylated upon activation [45], whereas proteins such as the heterotrimeric G_s protein must be depalmitoylated to be activated [13,46,47]. Since protein palmitoylation appears to have different effects on various palmitoylated substrates and at various cellular sites, multiple PAT enzymes may exist to palmitoylate specific proteins, and possibly to palmitoylate proteins in different cellular compartments. PAT enzymes that modify proteins in the endoplasmic reticulum, resulting in protein transport to the plasma membrane, for example, may be different from those at the plasma membrane that influence protein activity. A study by McCabe and Berthiaume [48] demonstrated differences in the cellular localization of proteins containing one myristoylation and one palmitoylation site versus proteins containing dual palmitoylation sites. Studies in yeast have also confirmed the subcellular localization of at least two PAT enzymes to the endoplasmic reticulum [23,49] and Golgi [24], each of which recognizes a unique peptide motif.

Using the IVP assay system described herein, the possibility of multiple PAT activities was evaluated directly by determining whether the palmitoylation of the two peptide substrates was differentially affected by various chemical treatments or changes in the assay parameters. As summarized in Table 3, treatment of MCF-7 membranes with detergent, NEM or heat clearly demonstrated that PAT activity towards the peptides could be

Table 3 Comparison of PAT activity toward FarnCNRas(NBD) and MyrGCK(NBD)

	FarnCNRas(NBD)	MyrGCK(NBD)	Significance of difference
Enzyme source			
MCF-7 cells	Slightly more activity	Slightly less activity	
Hep-G2 cells	Equal activity	Equal activity	
Wild-type 3T3 cells	No activity	More activity	$P < 0.05$
N-Ras-transformed 3T3 cells	Less activity	More activity	$P < 0.01$
K-Ras-transformed 3T3 cells	Less activity	More activity	$P < 0.001$
EJ-Ras-transformed 3T3 cells	Less activity	More activity	$P < 0.001$
Condition			
Ionic strength	Equal sensitivity	Equal sensitivity	
Assay temperature	Equal sensitivity	Equal sensitivity	
Non-ionic detergent	Less sensitive	More sensitive	$P < 0.001$
Thermal denaturation	More sensitive	Less sensitive	$P < 0.001$
DTT	Equal sensitivity	Equal sensitivity	
NEM	Less sensitive	More sensitive	$P < 0.001$
2-BP	Equal sensitivity	Equal sensitivity	
16C	Equal sensitivity	Equal sensitivity	

inhibited differentially. Furthermore, membranes isolated from wild-type NIH/3T3 cells demonstrated significant PAT activity towards MyrGCK(NBD), but had no detectable PAT activity towards FarnCNRas(NBD). Overall, these data suggest that independent PAT activities are present in cellular membranes to specifically palmitoylate each peptide substrate. This most likely reflects the existence of multiple PAT enzymes that palmitoylate specific motifs, such as the N-terminal motif on $p56^{lck}$ and the C-terminal motif on N-Ras.

With the identification and characterization of *AKR1* and *ERF2/ERF4* as PAT enzymes, researchers working with yeast have provided critical tools to start looking for the human homologues of these important enzymes. Roth et al. [24] have shown that the yeast protein Akr1p, a polytopic Golgi-membrane protein, is a PAT enzyme, and that Akr1p is required for palmitoylation of the casein kinase Yck2p *in vivo* and *in vitro*. Sequence analyses of Akr1p indicated that it contains ankyrin repeats and a Asp-His-His-Cys (DHHC) cysteine-rich domain. Homology studies have already identified over 120 proteins containing the essential DHHC cysteine-rich domain of Akr1p in *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans*, *Mus musculus*, *Homo sapiens* and *Arabidopsis thaliana*, with 23 DHHC-containing proteins in *H. sapiens* [24]. Although it is unlikely that all proteins containing this consensus sequence are PATs, the findings further support the hypothesis that multiple PAT enzymes exist within mammalian cells. For example, one protein that shares significant sequence and domain architectural homology with Akr1p, called HIP14, has already been identified in the human brain [50]. Although expression of HIP14 in *akr1*Δ yeast results in rescue of the endocytosis of the sex-hormone receptor, Ste3p, the function of HIP14 and its potential role in palmitoylation in human cells is yet unknown [50]. Sequence alignments using the yeast PAT enzyme, Erf2p, have also shown a high sequence and domain architectural homology with the putative human protein called new1-domain-containing protein (NEW1-CP; C.E. Ducker and C.D. Smith, unpublished work). The IVP assay described herein will be a useful tool to study the PAT activity of both human and yeast proteins and to characterize the specificity of such enzymes for particular protein substrates.

Not only is it important to characterize PAT activities to better understand the regulation of the palmitoylation of specific proteins, but it should also be important for the development of PAT inhibitors to be tested as anti-cancer therapeutics. Since many of the proteins modified by palmitate play important roles in signal transduction in both normal and tumour cells, it is possible that global inhibition of palmitoylation may have overwhelming effects on cell function. Instead, inhibition of individual PAT enzymes may allow selective targeting of signal-transduction pathways that allow tumour cells to proliferate continuously. For example, the palmitoylated proteins Ras and $p56^{lck}$ are products of oncogenes and have the ability to transform normal cells into tumour cells when aberrantly overexpressed or constitutively activated [51–56]. Oncogenic forms of Ras are found in 30 % of all human cancers and up to 90 % of pancreatic and 50 % of colon cancers [53,54]. An intriguing finding in these studies is that transformation of NIH/3T3 cells by any of the *ras* oncogenes results in greatly increased expression of PAT activity towards the FarnCNRas(NBD) peptide without any change in levels of PAT activity towards the MyrGCK(NBD) peptide (Table 3). Therefore, selective inhibition of the PAT that recognizes the C-terminal farnesyl palmitoylation motif may allow targeted therapy of *ras*-driven tumours. If compounds could target the PAT enzymes responsible for palmitoylating either Ras or $p56^{lck}$, these proteins could not be localized to the plasma membrane to confer activity of cell-proliferation or cell-survival pathways. The identification of substrate-specific PAT activities in this study implies that such targeted palmitoylation inhibition is possible, and that palmitoylation inhibition may be a novel target for the development of new anti-cancer therapeutics.

We would like to thank Dr Blake Peterson and Dr Stephen Creaser for their kind gift of the MyrGCK(NBD) peptide and Jean E. Copper for assisting with some of the PAT assays. Supported by National Institutes of Health grant RO1 CA 75248 (to C.D.S.).

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Received 11 October 2002/7 March 2003; accepted 1 April 2003
Published as BJ Immediate Publication 1 April 2003, DOI 10.1042/BJ20021598