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A fluorescence-based high performance liquid chromatographic method for the characterization of palmitoyl acyl transferase activity☆

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Abstract

Although protein palmitoylation is essential for targeting many important signaling proteins to the plasma membrane, the mechanism by which palmitoylation occurs is uncharacterized, since the enzyme(s) responsible for this modification remain unidentified. To study palmitoyl acyl transferase (PAT) activity, we developed an in vitro palmitoylation (IVP) assay using a fluorescently labeled substrate peptide, mimicking the N-terminal palmitoylation motif of proteins such as non-receptor Src-related tyrosine kinases. The palmitoylated and non-palmitoylated forms of the peptide were resolved by reverse-phase HPLC and detected by fluorescence. The method was optimized for PAT activity using lysates from the MCF-7 and Hep-G2 human tumor cell lines. The PAT activity was inhibited by boiling, reducing the incubation temperature, or adding 10 μM 2-bromopalmitate, a known palmitoylation inhibitor. This IVP assay provides the first method that is suitable to study all facets of the palmitoylation reaction, including peptide palmitoylation by PAT(s), depalmitoylation by thioesterases, and evaluation of potential palmitoylation inhibitors.

Keywords: Palmitoylation; Palmitoyl coenzyme A; Palmitoyl acyl transferase; Peptide; HPLC; Fluorescence analysis

Protein palmitoylation is a post-translational processing event in which a saturated 16-carbon fatty acid is covalently linked to a cysteine residue of a protein via a thioester bond [1]. Similar to other lipidation events, such as prenylation and myristoylation, palmitoylation is required for proper localization of proteins to subcellular structures [2–5]. Primarily, palmitoylation allows proteins to stably associate with the inner leaflet of the plasma membrane [6,7]. Unlike other covalent lipidation events, palmitoylation is a dynamic process in which proteins can cycle between palmitoylated and non-palmitoylated forms [8,9]. Therefore, of the various lipid modifications of proteins, palmitoylation has the greatest potential to be regulated by thioesterases that act to depalmitoylate proteins [10–12] and palmitoyl acyl transferases (PAT)[1] that catalyze protein palmitoylation reactions. Although the mechanisms by which proteins are prenylated [13] and myristoylated [14–16] have been clearly defined, the mechanism by which proteins become palmitoylated is not well understood due to the lack of biochemical characterization of PAT(s).

The subset of cellular proteins that undergo palmitoylation includes important signaling proteins, including non-receptor Src-related tyrosine kinases (Fyn, Lck, and Yes) [17–19], α-subunits of heterotrimeric G-proteins [20], and H-/N-Ras [21,22], as well as important

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[1] *Abbreviations used:* HPLC, high-performance liquid chromatography; IVP, in vitro palmitoylation; NBD, 7-nitrobenz-2-oxa-1,3-diazol-4-yl; PMSF, phenylmethylsulfonyl fluoride; PAT, palmitoyl acyl transferase.

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transmembrane receptors such as the β_2-adrenergic receptor [23] and several G protein-coupled receptors [24–26]. Since many of these cellular proteins require palmitoylation for proper cellular localization and optimal activity, understanding the mechanism of protein palmitoylation is essential for a more complete understanding of the protein function and the regulation of cell signaling events.

The general mechanism for protein palmitoylation is currently under debate, with evidence supporting both enzymatic [22,27,28] and non-enzymatic [29] palmitoylation depending on the protein substrate. Developing a palmitoylation assay sensitive enough to distinguish between non-enzymatic and enzymatic protein palmitoylation is crucial to facilitate the characterization of PAT. In this study, we describe such a system using highly sensitive fluorescence-based HPLC analyses to measure the PAT activity with an NBD-labeled peptide substrate that mimics the palmitoylatable N-terminal sequence of Src-related kinases [30].

Materials and methods

Materials

The palmitoylatable peptide substrate was synthesized as previously described [30]. Palmitoyl coenzyme A (CoA) and 2-bromopalmitate were from Sigma (St. Louis, MO). HPLC-grade water was from Honeywell International, Burdick and Jackson (Muskegon, MI). HPLC-grade acetonitrile was from EM Science (Gibbstown, NJ). The C_4-HPLC column and HPLC-grade methanol were from Fisher Scientific (Fair Lawn, NJ). 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).

Cell culture

Hep-G2 cells (ATCC HB-8065) were maintained in DMEM containing 10% fetal bovine serum, 50 μg/ml gentamicin, and 1 mM sodium pyruvate at 37 °C in an atmosphere of 5% CO_2 and 95% air. MCF-7 (ATCC HTB-22), T24 (ATCC HTB-4), and Jurkat (ATCC TIB-152) cells were maintained in RPMI media containing 10% fetal bovine serum and 50 μg/ml gentamicin at 37 °C in an atmosphere of 5% CO_2 and 95% air. NIH/3T3 wild-type cells (ATCC CRL-1658) were maintained in DMEM containing 10% calf serum, 50 μg/ml gentamicin, and 1 mM sodium pyruvate at 37 °C in an atmosphere of 5% CO_2 and 95% air.

Preparation of subcellular fractions

To determine the optimal subcellular fraction for use in the in vitro palmitoylation (IVP) assay, cells were fractioned as described in Smith et al. [31]. Briefly, MCF-7 or Hep-G2 cells were grown to about 70% confluency in 150 mm tissue culture dishes and collected by centrifugation. The cells were swollen with 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 the nuclei and debris. The nuclei and debris pellet were collected as the pellet fraction. The supernatant from the low-speed centrifugation was then ultracentrifuged at 100,000*g* for 1 h at 4 °C. The resulting pellet from this ultracentrifugation was resuspended in 400 μl lysis buffer and collected as the membrane fraction. The supernatant from the ultracentrifugation was also collected as the cytosolic fraction. Each subcellular fraction was assayed for protein concentration with a fluorescamine assay [32] and used in IVP assays, immediately following their preparation.

In vitro palmitoylation assay method

The palmitoylatable peptide substrate was stored as the *t*-butyl-disulfide precursor and deprotected immediately before use by incubation with 2.75% β-mercaptoethanol in 10 mM Tris/DMSO buffer at 55 °C for 15 min with agitation. The standard IVP assay mixture contains 10 μM deprotected peptide, 20 μM palmitoyl CoA, 50 μg protein, and acylation buffer (50 mM citrate, 50 mM phosphate, 50 mM Tris, and 50 mM CAPS at pH 7.2) in a total volume of 100 μl. The IVP assay was performed by first incubating the peptide with the enzyme source in acylation buffer for 8 min at 37 °C with agitation. Palmitoyl CoA was then added to the assay mixture to start the palmitoylation reaction and incubation was continued at 37 °C for an additional 7.5 min. The assay was stopped upon addition of the assay mixture to 1.2 ml of CH_2Cl_2:methanol:water (2:1:1), which not only stops the reaction but also extracts the peptide into the organic phase. The organic phase was collected by low-speed centrifugation and the extraction was repeated two times. The CH_2Cl_2 fractions were combined, dried under N_2, and stored at −20 °C until analyzed as indicated below.

HPLC method

The HPLC unit consisted of the Beckman Coulter System Gold and Waters 470 Scanning Fluorescence Detector. The assay extracts were dissolved in 25 μl DMSO and peptides were resolved on a reverse phase,

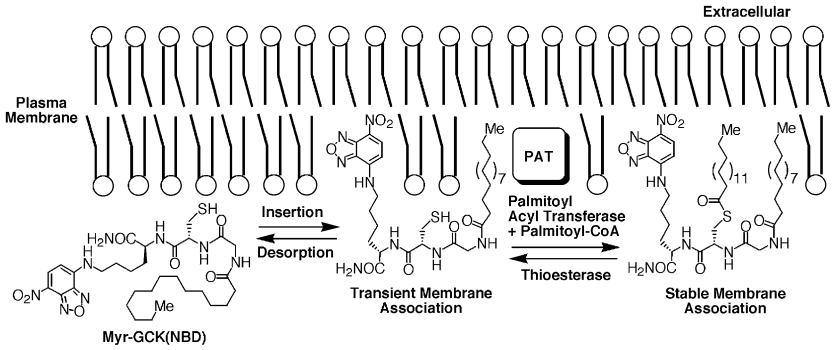

Fig. 1. Schematic of incorporation of the Myr-GCK(NBD) peptide into the plasma membrane. With the single myristoylation modification, the peptide is only transiently associated with the inner leaflet of the membrane. PAT then acts to palmitoylate the peptide, causing stable association at the membrane. This reaction can be reversed by removal of the palmitate moiety by thioesterase activity.

wide pore butyl (5 μM, 300 Å, 4.6 × 250 mm) column using an acetonitrile gradient with a flow rate of 1 ml/min. Initially, the mobile phase was maintained as water/50% CH_3CN/0.1% TFA for 5 min, followed by a 5 min linear gradient from 50 to 100% CH_3CN. The mobile phase was then maintained at 100% CH_3CN for 10 min, followed by a linear gradient from 100 to 50% CH_3CN over 5 min. The NBD-labeled peptide was detected by its fluorescence at its optimal excitation and emission wavelengths of 465 and 531 nm, respectively. The limit of detection was about 4 ng NBD-labeled peptide, as determined with NBD-C_{12}-HPC used as an internal standard. The percentage of palmitoylated peptide in each assayed sample was calculated by dividing the peak area corresponding to the palmitate-modified peptide by the total peak area corresponding to both unmodified and palmitate-modified peptides.

Results

Resolution of NBD-labeled peptides

Palmitoylated proteins and peptides are susceptible to both palmitoylation by PAT and depalmitoylation by thioesterase as shown in Fig. 1. An assay that directly measures peptide palmitoylation was developed using an NBD-labeled peptide substrate, termed Myr-GCK(NBD), containing an N-terminal myristoylated glycine, followed by a palmitoylatable cysteine residue. Peptide palmitoylation could be quantified by HPLC analysis with fluorescence detection of the NBD-tag, as demonstrated in the HPLC chromatographs in Fig. 2. Incubating 10 μM peptide at 37 °C in acylation buffer (pH 8.0) alone for 5 min (solid-line trace) resulted in two peaks at retention times of 10.7 and 13. 3 min (peaks labeled A and B, respectively). Peak A with a retention time of 10.7 min represents the deprotected peptide, whereas peak B with a retention time of 13.3 min represents a disulfide linked peptide dimer. When incubating 10 μM peptide with 100 μM palmitoyl CoA (broken-line trace) for 5 min at 37 °C in acylation buffer (pH 8.0), a new peak eluted from the HPLC column with a retention time of 14.3 min (peak labeled C in Fig. 2). The longer retention time suggested that peak C is the palmitate-modified form of the Myr-GCK(NBD) substrate. Analysis of peak C by high-resolution mass spectrometry with negative ion electrospray ionization (performed by The Scripps Research Institute, La Jolla, CA) verified that it was the palmitoylated form of the Myr-GCK(NBD) peptide with an expected mass of 916 (data not shown).

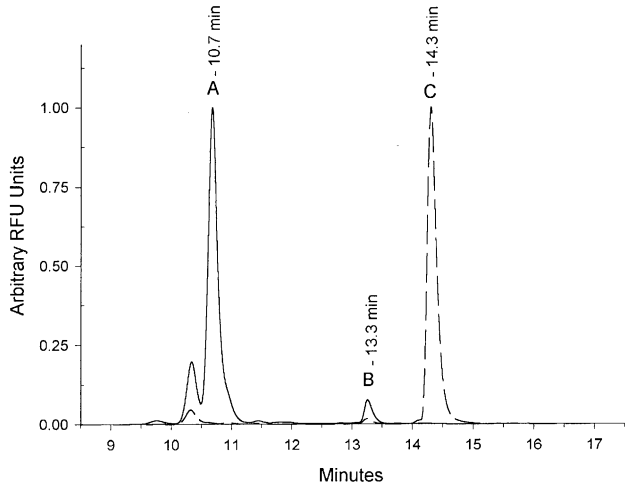

Fig. 2. Chromatographs of the Myr-GCK(NBD) peptide and the palmitoylated Myr-GCK(NBD) peptide. The solid-line trace represents 10 μM peptide incubated at 37 °C in acylation buffer (pH 8.0) for 5 min in the absence of palmitoyl CoA. The broken-line trace represents the chromatograph of autoacylation of 10 μM peptide with 100 μM palmitoyl CoA for 5 min at 37 °C in acylation buffer (pH 8.0). The peptide was extracted out of the reaction mix as described in Materials and methods. Peak A, deprotected Myr-GCK(NBD); peak B, disulfide-linked Myr-GCK(NBD); peak C, palmitate-modified Myr-GCK(NBD).

Optimization of palmitoylation conditions

A long-standing difficulty in performing in vitro protein palmitoylation assays is distinguishing chemical palmitoylation (autoacylation) from enzymatic palmitoylation. Autoacylation of the Myr-GCK(NBD) peptide substrate is favored when the pH and/or palmitoyl CoA concentration of the palmitoylation reaction are increased, as demonstrated in Fig. 3. The dependence of peptide palmitoylation on assay pH and palmitoyl CoA concentration was evaluated by performing chemical palmitoylation reactions in which 10 μM Myr-GCK(NBD) peptide was incubated with varying palmitoyl CoA concentrations at various pHs for 5 min at 37 °C. The results of this assay revealed significant increases in peptide autoacylation as the assay pH was increased from 6.6 to 8.0. The increased level of peptide palmitoylation at pH 8.0 was expected, however, since peptide palmitoylation is highly favored at pHs close to the pK_a of cysteine [33]. Similar to results observed with increasing pH assay conditions, Myr-GCK(NBD) peptide autoacylation was also significantly increased as palmitoyl CoA concentrations increased from 10 to 100 μM. Since chemical palmitoylation of the Myr-GCK(NBD) peptide is sensitive to changes in pH and palmitoyl CoA concentrations, IVP assay conditions had to be selected that would allow distinction between autoacylation and enzymatic peptide palmitoylation. Therefore, the conditions selected for further IVP assay development consisted of a pH of 7.2, which remains physiologically relevant, and a palmitoyl CoA concentration that is 2-fold higher than that of the peptide. This palmitoyl CoA concentration corresponds with cellular concentrations of acyl CoAs, which range from nanomolar to 160 μM for free and bound acyl CoAs, respectively [34]. The optimized IVP assay conditions allowed for clear distinction between non-enzymatic and enzymatic palmitoylation.

Characterization of enzymatic palmitoylation

Enzymatic palmitoylation was apparent when 50 μg protein from membrane or pellet fractions collected from MCF-7 human breast cancer or HepG2 human hepatocellular carcinoma cells were used as PAT sources. Time course studies demonstrated that the optimal incubation time for the IVP assay included a preincubation of peptide with the enzyme source for 8 min, followed by addition of palmitoyl CoA for an additional 7.5 min incubation (data not shown). The results of the optimized IVP assay show increased peptide palmitoylation in the presence of either Hep-G2 or MCF-7 pellet or membrane fractions (Fig. 4). The level of peptide palmitoylation with Hep-G2 pellet and membrane enzyme sources was 42 and 60%, respectively, which is a significant increase over autoacylation ($p < 0.05$). The level of peptide palmitoylation with MCF-7 pellet and membrane enzyme sources was 52 and 60%, respectively, which is also a significant increase over autoacylation ($p < 0.001$). Interestingly, the cytosolic fractions collected from these cells decreased the accumulation of palmitoylated peptide compared to autoacylation controls (Fig. 4), indicating that thioesterases are present in this fraction. Although pellet fractions demonstrated significant increases in peptide palmitoylation, further characterization revealed marked

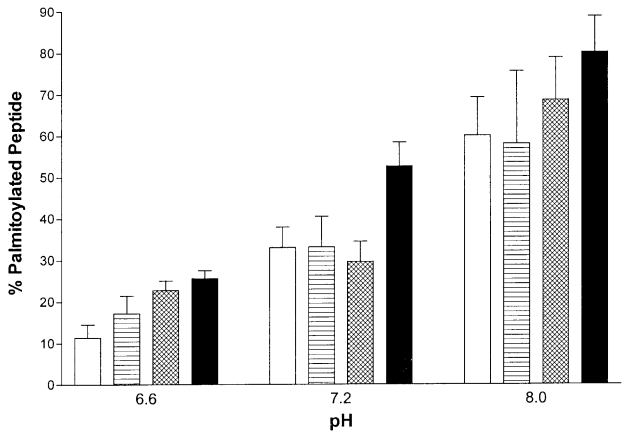

Fig. 3. Autoacylation of the Myr-GCK(NBD) peptide substrate under various pH and palmitoyl CoA concentration conditions. Peptide (10 μM) was incubated with 10 μM (□), 20 μM (▤), 50 μM (▨), or 100 μM (■) palmitoyl CoA at the indicated pHs for 5 min at 37 °C. The percentage of palmitoylated peptide was then determined as described in Materials and methods. Values represent means ± SD percentages of the palmitoylated peptide. Results from one of the three similar experiments are shown.

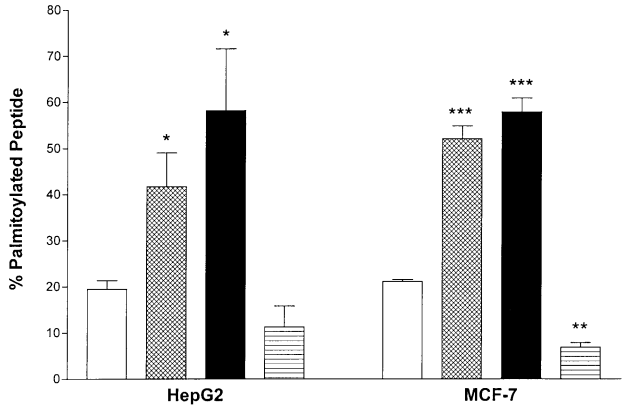

Fig. 4. Palmitoylation of the Myr-GCK(NBD) peptide using the optimized IVP assay. Peptide (10 μM) was preincubated with no enzyme (□), HepG2 or MCF-7 pellet (▨), membrane (■), or cytosol (▤) fractions for 8 min and then incubated with 20 μM palmitoyl CoA for an additional 7.5 min at 37 °C before analysis as described in Materials and methods. Values represent means ± SD percentages of palmitoylated peptide in quadruplicate samples. Results from one of the five similar experiments are shown. ($^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared to autoacylation).

variability in the PAT activity with this enzyme source. In contrast, PAT activity in the membrane fraction was highly consistent. Therefore, only membrane fractions were used as the source of PAT in further experiments.

The dependence of peptide palmitoylation on the membrane protein concentration in the IVP assay was evaluated (Fig. 5). To determine the concentration at which the optimal palmitoylation response could be elicited, a range of protein concentrations from 1 to 100 μg were assayed. As the MCF-7 and Hep-G2 PAT membrane concentrations were increased from 1 μg protein to 5 μg protein, no significant increases in peptide palmitoylation were observed above the peptide autoacylation. Concentration-dependent increases in peptide palmitoylation above autoacylation were observed, however, as Hep-G2 and MCF-7 PAT membrane concentrations were increased from 10 to 100 μg protein in the IVP assay. Peptide palmitoylation was significantly increased over autoacylation with membrane concentrations of at least 25 μg protein for Hep-G2 and 50 μg protein for MCF-7 membrane fractions. As the Hep-G2 membrane concentration increased from 50 to 100 μg protein, the level of peptide palmitoylation began to plateau at about 60% palmitoylated peptide substrate, indicating that the PAT enzyme source may reach a level of saturation at a concentration of 100 μg protein. Since PAT activity began to plateau at protein concentrations exceeding 50 μg protein, a concentration of 50 μg protein was used in further experiments.

To verify that the peptide palmitoylation observed in Fig. 4 is due to enzymatic activity rather than non-specific interactions with components in the membrane fraction, we performed IVP assays under enzymatically unfavorable conditions and examined how these conditions affected Myr-GCK(NBD) peptide palmitoylation. Enzymatic activity was first evaluated using denatured protein from membrane fractions that had been boiled at 95 °C for 5 min, prior to the assay as the PAT enzyme source. As demonstrated in Fig. 6, boiling the Hep-G2 or MCF-7 membranes, prior to the IVP assay significantly reduced palmitoylation of the peptide substrate to levels equivalent to autoacylation, indicating denaturation of PAT. The enzymatic activity was also evaluated by performing the IVP assay incubations at 4 °C. Compared to enzymatic peptide palmitoylation observed under normal thermal conditions, enzymatic palmitoylation of the Myr-GCK(NBD) peptide was significantly inhibited at 4 °C. The level of peptide palmitoylation at 4 °C also decreased below peptide autoacylation levels, indicating that the rates of both enzymatic and chemical palmitoylation decrease with decreasing temperature.

The PAT activity measured by Myr-GCK(NBD) palmitoylation was also pharmacologically assessed using a known palmitoylation inhibitor, 2-bromopalmitate (Fig. 7). When Hep-G2 or MCF-7 membranes were incubated with 1% ethanol, the level of peptide palmitoylation reached 36 and 41%, respectively, which was significantly increased above the level of peptide autoacylation of 19% (confidence of $p < 0.005$). In the presence of 10 μM of 2-bromopalmitate, palmitoylation of the peptide substrate was significantly decreased to levels equivalent to peptide autoacylation, 14% with Hep-G2 and 17% with MCF-7 membranes (confidence of $p < 0.001$). These results demonstrate further that the membrane-catalyzed peptide palmitoylation occurs through PAT.

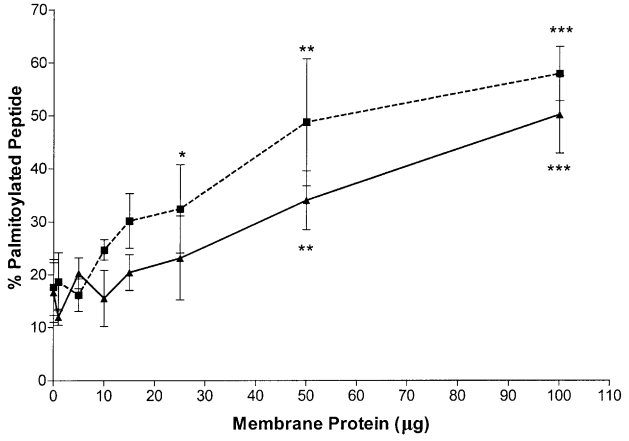

Fig. 5. Dependence of Myr-GCK(NBD) peptide palmitoylation on membrane protein concentration. The IVP assay was performed with increasing amounts of Hep-G2 membrane (■, dotted line) or MCF-7 membrane (▲, solid line) concentrations. Values represent means±SD percentages of the palmitoylated peptide in quadruplicate samples. Results from one of the three similar experiments are shown. ($^{*}p < 0.05$, $^{**}p < 0.005$, and $^{***}p < 0.001$ compared to autoacylation).

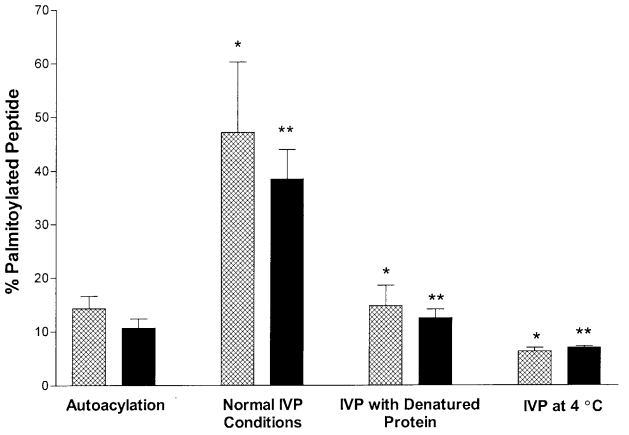

Fig. 6. Effects of enzyme deactivation on Myr-GCK(NBD) peptide palmitoylation. IVP assays were performed with membranes that had been boiled for 5 min, prior to the assay or in which the incubation temperature was set to 4 °C. [Hep-G2 membrane (▒); MCF-7 membrane, (■).] Values represent means ± SD percentages of the palmitoylated peptide in quadruplicate samples. Results from one of the two similar experiments are shown ($^{*}p < 0.05$, $^{**}p < 0.005$).

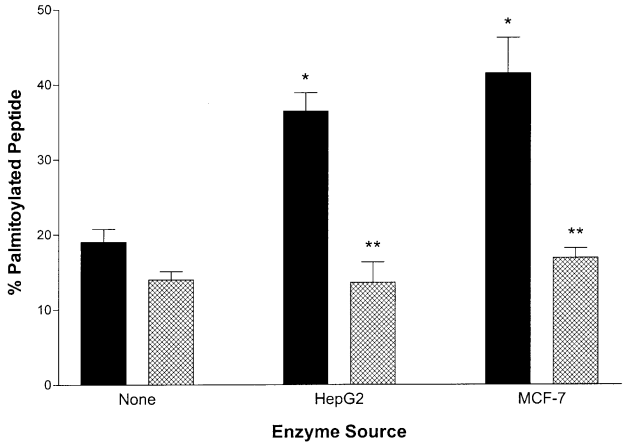

Fig. 7. Effects of 2-bromopalmitate on Myr-GCK(NBD) palmitoylation. Assays were conducted in the presence of ethanol (vehicle control, (■)) or 10 μM 2-bromopalmitate (▒). Values represent means ± SD percentages of the palmitoylated peptide in quadruplicate samples. Results from one of the two similar experiments are shown. ($^{*}p < 0.005$ compared to autoacylation, $^{**}p < 0.001$ compared to vehicle-control).

Survey of PAT activity in membrane fractions of different cell lines

In addition to Hep-G2 and MCF-7 cells, PAT activity was assayed in T24 human bladder carcinoma cells, Jurkat human T cell leukemia cells, and NIH/3T3 mouse fibroblasts (Table 1). In contrast with the MCF-7 and Hep-G2 cell lines, no significant enzymatic palmitoylation activity was observed with T24, Jurkat, or NIH/3T3 membrane fractions in excess of autoacylation of the peptide. Based on these findings, it appears that either the PAT activity within T24, Jurkat, and NIH/3T3 membrane fractions is much lower than in MCF-7 and Hep-G2 cells, or thioesterase activity within these membrane fractions is much higher.

Discussion

In an attempt to better characterize the protein palmitoylation reaction and PAT activity, we have developed a non-radioactive, highly sensitive in vitro palmitoylation assay using HPLC analysis with fluorescence detection. This assay is one of the first tools that are capable of directly measuring the PAT activity. Analysis of peptide palmitoylation in the IVP assay under unfavorable enzymatic conditions clearly demonstrates that PAT activity can be quantified and distinguished from non-enzymatic peptide palmitoylation activity. The most convincing evidence that this assay measures enzymatic palmitoylation is the observed inhibitory effect of 2-bromopalmitate on the palmitoylation of the peptide substrate. Since the IVP assay is capable of consistently and sensitively monitoring the PAT activity, this assay will be a useful tool for the characterization of PAT activity and the palmitoylation reaction.

Table 1
In vitro palmitoylation of the Myr-GCK(NBD) peptide using membranes from various cell lines

Enzyme source	% Palmitoylated peptide[a]
None	19 ± 4
HepG2	58 ± 27
MCF-7	60 ± 13
Jurkat	27 ± 6
NIH/3T3 (WT)	23 ± 2
T24	18 ± 10

[a] Hep-G2, MCF-7, Jurkat, NIH/3T3, and T24 membrane fractions were assayed for PAT activity using the standard IVP assay. Values represent means ± SD of the peptide palmitoylated in quadruplicate samples.

Success in attempts to characterize protein palmitoylation has been limited for many reasons, including the lack of PAT identification and cloning, the loss of palmitate groups from proteins during purification processes, rapid turnover of palmitate bound to proteins, and the uncharacterized stoichiometry of the palmitoylation reaction [9]. Many of these attempts to characterize protein palmitoylation were performed by isotopic labeling of cellular proteins or peptide constructs with [^{3}H]palmitate, [^{3}H]palmitoyl CoA, or [^{125}I]palmitoyl CoA [7,17,22,35]. However, these techniques are disadvantageous for several reasons: the isotopes become diluted with endogenous palmitate during cell labeling; the palmitoyl CoA substrate may bind to or be metabolized by proteins that are not involved in the palmitoylation reaction, such as acyl binding proteins; the palmitate group can be released from proteins, prior to analysis; and visualization of isotope-labeled proteins by autoradiography often requires exposure to film for weeks or months [9]. Perhaps, the greatest disadvantage of characterizing enzymatic palmitoylation with isotope labeling experiments is that these techniques do not easily distinguish between non-enzymatic and enzymatic palmitoylation, making both the characterization of PAT and evaluation of palmitoylation of specific substrate proteins very difficult. We can now overcome many of these problems using the assay described herein to unambiguously identify a specific fluorescently labeled palmitoylated peptide, accurately quantify the amounts of palmitate-modified substrate versus non-palmitoylated substrate, and distinguish non-enzymatic palmitoylation from enzymatic palmitoylation.

Previous studies with intact Jurkat cells [30] demonstrated that the Myr-GCK(NBD) peptide is localized to the plasma membrane when it is palmitoylated, as illustrated in Fig. 1. In this model, the myristoylated peptide only transiently associates with the membrane, whereas palmitoylation by PAT is required for stable

localization of the peptide at the inner leaflet of the plasma membrane. Stable membrane localization of the peptide can be regulated further by thioesterases, which cleave the palmitate from the peptide, thereby reducing the peptide's affinity for the plasma membrane. Since the palmitoylation state of the peptide is regulated by both thioesterases and PAT, the peptide may cycle between a non-palmitoylated and palmitoylated form during the IVP reaction. Since cells contain both PAT and thioesterase activity, the extent of Myr-GCK(NBD) palmitoylation is likely determined by the relative amounts of these enzymes in the test sample. In the presence of both PAT and thioesterases, the time for which the palmitate is linked to the peptide, as with other protein substrates, may be relatively short. For example, the half-lives of the palmitate bound to N-Ras and $G_{\alpha s}$ proteins are about 20 and 30 min, respectively [36,37], whereas the half-life of palmitate bound to β_2-adrenergic receptors ranges between 2 and 9 min [12,38]. Therefore, another problem in the development of an in vitro palmitoylation assay has been the need to try to trap the product in its palmitate-modified form. Using the IVP assay described in this study, palmitoylation of the peptide substrate can be monitored and accurately measured to study the dynamic processing event and therefore to observe the actions of PAT(s) and thioesterases. This dynamic processing is a possible limitation of the assay of PAT activity. For example, thioesterase activity was clearly present in the cytosolic fractions of the MCF-7 and Hep-G2 cells, as demonstrated by the decrease in peptide palmitoylation below that of the no-enzyme control. Thioesterases may even slightly contaminate membrane fractions, since the palmitoylation of Myr-GCK(NBD) reached a maximum of only 65–75% with this assay. Thus, it should be recognized that the presence of thioesterase activity within the test sample may lead to underestimation of PAT activity.

Signaling proteins that are modified by palmitoylation include examples of N-myristoylated proteins and C-farnesylated proteins [2,6]. It is not yet clear if multiple PAT enzymes exist to palmitoylate the distinct motifs of these proteins. The IVP assay described herein will be a useful tool to determine whether motif-specific PAT enzymes are present in the cells. In the present study, enzymatic palmitoylation of the Myr-GCK(NBD) peptide substrate, which mimics the N-terminus of non-receptor Src-related tyrosine kinases, was characterized. Comparing the palmitoylation parameters of this substrate with those of a second peptide substrate, e.g., a farnesylated peptide mimicking the C-terminus of H-Ras [42], will be highly useful in determining if the same enzyme is responsible for the palmitoylation of both classes of substrate proteins.

Although not yet molecularly characterized, PAT(s) have the potential to be an important therapeutic target. Since many proteins that undergo palmitoylation are oncogenic, such as H-/N-Ras and Lck [43], PAT enzymes(s) may play a significant role in tumorigenesis and therefore should be characterized as a novel target for the development of anti-cancer therapeutics. Other lipid-modifying enzymes, such as farnesyltransferase, have been recognized as important targets for the development of chemotherapeutic agents. Farnesyltransferase inhibitors have been actively pursued as novel anti-cancer therapeutics and have demonstrated chemotherapeutic success by causing tumor regression and reversing malignant phenotypes in preclinical models [44]. A number of farnesyltransferase inhibitors are currently undergoing clinical trials.

Identification and characterization of protein palmitoyl thioesterases have provided some insight into the importance of protein palmitoylation for cell function. For example, thioesterase deficiencies have been linked to neuronal diseases such as infantile neuronal ceroid lipofuscinosis [39]. Overexpression of thioesterases has been shown to protect cells from undergoing drug-induced apoptosis [40], whereas inhibition of thioesterases has been shown to increase the cellular susceptibility to programmed cell death [41]. These studies implicate the regulation of palmitoylation as a crucial process in the maintenance of cell survival. Although the physiological relevance of thioesterases has been studied, it will be equally important to determine the role of PAT(s) in cell function.

The potential for palmitoylation to play a role in tumorigenesis or other diseases states has not yet been identified or realized, primarily due to the lack of biochemical characterization of the palmitoylation event. The IVP assay should be instrumental for the detailed characterization of PAT activity as the first step to determine its physiological relevance and the role of palmitoylation in normal and disease states.

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