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Use of micellar electrokinetic chromatography to measure palmitoylation of a peptide

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ABSTRACT

Palmitoylation is the thioester linkage of the fatty acid, palmitate (C16:0), to cysteine residues on a protein or peptide. This dynamic and reversible post-translational modification increases the hydrophobicity of proteins/peptides, facilitating protein–membrane interactions, protein–protein interactions and intracellular trafficking of proteins. Manipulation of palmitoylation provides a new mechanism for control over protein location and function, which may lead to better understanding of cell signaling disorders, such as cancer. Unfortunately, few methods exist to quantitatively monitor protein or peptide palmitoylation. In this study, a capillary electrophoresis-based assay was developed, using MEKC, to measure palmitoylation of a fluorescently-labeled peptide *in vitro*. A fluorescently-labeled peptide derived from the growth-associated protein, GAP-43, was palmitoylated *in vitro* using palmitoyl coenzyme A. Formation of a doubly palmitoylated GAP-peptide product was confirmed by mass spectrometry. The GAP-peptide substrate was separated from the palmitoylated peptide product in less than 7 min by MEKC. The rate of *in vitro* palmitoylation with respect to reaction time, GAP-peptide concentration, pH, and inhibitor concentration were also examined. This capillary electrophoresis-based assay for monitoring palmitoylation has applications in biochemical studies of acyltransferases and thioesterases as well as in the screening of acyltransferase and thioesterase inhibitors for drug development.

1. Introduction

Palmitoylation is a post-translational modification in which a palmitate (C16:0) group is added *via* a thioester bond to a cysteine in a protein. This dynamic and reversible modification increases the hydrophobicity of proteins, thereby increasing interactions with other hydrophobic moieties, such as the plasma membrane. Palmitoylation cycling plays a role in cell signaling by promoting the movement of proteins to different sites of action within the cell [1–9]. Palmitoylation is also implicated in the regulation of protein trafficking [10–14], as well as promoting protein–protein interactions [15,16] and modulating enzyme activity [17–19]. For example, palmitoylation of the growth-associated protein, GAP-43, helps to direct the protein to the plasma membrane, where it is involved in neuronal growth and spreading, particularly the extension and branching of neuronal axon tips, or growth cones [20–23]. GAP-43 is palmitoylated during axonal growth cone extension and branching, but is not palmitoylated during growth cone maturation [24,25]. Interestingly, GAP-43 protein expression in axons remains unchanged between the growth and the final development of mature synapses [26]. Palmitoylation, therefore, is the switch between axon growth and maturation, rather than absolute protein expression. In the case of neuronal growth and GAP-43, palmitoylation acts as an efficient cellular mechanism to control the construction and remodeling of a system as dynamic and plastic as a neuronal synapse in the developing brain.

Palmitoylation of proteins and peptides is commonly monitored by isotope radiolabeling techniques [27]. Protein or peptide substrates are incubated with tritiated palmitoyl coenzyme A ([^{3}H]palmitoyl-CoA) with or without enzymes. The transfer of the [^{3}H]palmitoyl moiety onto the substrate is monitored using SDS-PAGE separations and detected by gel staining techniques. While this technique is widely used, it has limitations. Radiolabeling is laborious and sample intensive. The radioactive palmitoyl-CoA and the generated radioactive waste are expensive. The time to perform the full assay requires at least one week. Unfortunately, this technique is qualitative, limiting its use in kinetic studies. In addition, because only the palmitoyl group is radiolabeled, this technique cannot be used to quantitatively study the dynamic, and equally important, de-palmitoylation event.

Recently, engineered peptides, selected for their structural similarity to native palmitoylated proteins, have been synthesized and used to study palmitoylation using high performance liquid chromatography (HPLC) [28–30]. Fluorescently-labeled tripeptide

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substrates representing the palmitoylation motif of ras proteins were used to characterize the activity of palmitoylation enzymes, known as acyltransferases, extracted from numerous cancer cell lines. Using low-retention columns (HPLC-C_4) and fluorescence detection, a palmitoylated ras tripeptide substrate was separated from non-palmitoylated substrate. Monitoring palmitoylation by HPLC offers the benefits of lower sample volumes (μL) and quicker analysis time (several hours); however, HPLC utilizes large volumes of solvent with the attendant costs of waste disposal and the sample size remains too large for assays in which only small amounts of biologic reagents are available.

Relative to HPLC, capillary electrophoresis (CE) has many advantages including very small samples sizes, ranging from pL to nL, detection limits as low as zeptomoles, and separation times of seconds to minutes, which are ideal conditions for monitoring dynamic chemical and biological processes in single cells or small groups of cells [31]. CE-based separation of fluorescently-labeled peptides has been used to develop novel biochemical assays for monitoring and measuring the dynamics of different post-translational modifications, including phosphorylation [32–34], S-nitrosylation [35] and farnesylation [36–38]. Fluorescently-labeled peptides, as opposed to proteins, are easier to load into cells, can be designed to react with specific enzymes *in vivo*, and are readily separated by CE, suggesting that they make ideal substrates for the study of enzymatic processes, such as palmitoylation, in single cells. Increased interest in measuring and controlling protein palmitoylation in diseases, such as cancer, necessitates the development of novel substrates (fluorescently-labeled peptides, for instance) and assays with increased throughput relative to current assays to study palmitoylation. CE, coupled with the use of fluorescently-labeled substrates, is ideally suited to monitoring dynamic processes such as palmitoylation in single cells [31].

This paper reports on the development of a CE-based assay for monitoring the palmitoylation of a basic peptide utilizing micellar electrokinetic chromatography (MEKC) and laser-induced fluorescence (LIF) detection. A 9-amino acid peptide sequence, corresponding to the palmitoylated portion of the GAP-43 protein, was selected, synthesized, and fluorescently-labeled for use in the palmitoylation assay. The GAP-peptide substrate was non-enzymatically palmitoylated and a method was developed to separate the palmitoylated product from the non-palmitoylated substrate. Parameters affecting the rate of non-enzymatic palmitoylation, including time dependence, addition of a competitive inhibitor, and pH, were also examined.

2. Experimental

2.1. Reagents

All tissue culture materials were obtained from Gibco/Invitrogen (Carlsbad, CA, USA). Palmitoyl coenzyme A (palmCoA), 2-bromohexadecanoic acid (2-bromopalmitate, 2-BP) Tris base, tetraborate, cetyl trimethylammonium bromide (CTAB), sodium dodecyl sulfate (SDS), sodium deoxycholate (SDC) and Triton X were used as purchased from Sigma–Aldrich (St. Louis, MO, USA). Fused silica capillary tubing (i.d. 50 μm) was obtained from Polymicro Technologies, Inc. (Phoenix, AZ, USA). All other reagents and materials were purchased from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Peptide synthesis

A fluorescently-labeled version of the GAP-peptide substrate (HiLyteFluor488-MLCCMRRTK) was synthesized, fluorescently-labeled, and purified by AnaSpec, Inc. (San Jose, CA, USA). HiLyteFluor488 is a relative of fluorescein with increased pH stability. The underlined cysteine residues correspond to the sites of palmitoylation in this peptide. GAP-peptide was used as received from AnaSpec, Inc. Stock solutions (1 mM) of GAP-peptide were prepared by dissolving the peptide in distilled, deionized water (ddH_2O) and were stored at −20 to −70 °C prior to use.

2.3. MALDI-MS

A MALDI-TOF instrument (Voyager DE STR, PerSeptive Biosystems, Framingham, MA, USA) using a 337 nm nitrogen laser, coupled to a 2.0 m linear flight tube, with a 3.0 m effective length in reflector mode, was used to confirm the relative purity of the GAP-peptide sample and the molecular weight of the GAP-peptide and the palmitoylated GAP-peptide product. The samples were prepared in a DHB matrix (20 mg/mL 2,5-dihydroxybenzoic acid in 50% methanol:0.1% trifluoroacetic acid in ddH_2O) prior to MALDI-MS measurement.

2.4. Production of a palmitoylated standard peptide

A standard of the fully palmitoylated GAP-peptide was generated by mixing 100 μM GAP-peptide with 1 mM palmCoA in acylation buffer (50 mM HEPES buffer (pH 7), 1 mM EGTA, 1 mM $MgCl_2$, 10 mM NaCl, 130 mM KCl, and 1 mM dithiothreitol (DTT)), and incubating for 2–4 h at 37 °C. MALDI-MS and CE were used to confirm that the palmitoylation of GAP-peptide by palmCoA went to completion. Reaction mixtures of GAP-peptide and palmCoA containing only the doubly palmitoylated peptide product (referred to as palm-GAP-peptide) were used as palmitoylated peptide standards without purification.

2.5. Measurement of fluorescence spectra

To examine the effect of palmitoylation on the fluorescence characteristics of the peptide, the fluorescence spectra of 1 μM GAP-peptide and 1 μM palm-GAP-peptide product were measured in neutral aqueous buffer (acylation buffer, pH 7) and in the hydrophobic MEKC electrophoresis buffer (100 mM Tris, 10 mM CTAB, 10% EtOH, pH 8.5). Spectra from duplicate samples of the four different mixtures loaded onto a single 96-well plate were measured using a microplate reader (Molecular Devices M5, Sunnyvale, CA, USA) with an excitation wavelength of 473 nm (9 nm excitation bandwidth), corresponding to the excitation wavelength used in the capillary electrophoresis system. Relative peak areas for palm-GAP-peptide and GAP-peptide were generated by integrating each spectrum between 510 and 560 nm (15 nm emission bandwidth), which corresponded to the band pass filter of the CE system. All spectra were collected under identical instrumental operating conditions.

2.6. In vitro palmitoylation assay (non-enzymatic palmitoylation)

Multiple electrophoretic buffers were tested for separation of GAP-peptide from palm-GAP-peptide. The electrophoretic buffers contained either Tris (100 mM, pH 8.5) or tetraborate (25 mM, pH 8.5) combined with a single surfactant, either SDS (25 mM), SDC (20 mM), Triton X (1%), or CTAB (10 mM). Organic modifiers, including urea (3 M), acetonitrile (10%), methanol (10%) and ethanol (10%) were also added to the electrophoretic buffers to aid in separation.

The time course of palmitoylation of GAP-peptide *in vitro* was measured by sampling a mixture of 10 μM GAP-peptide and 100 μM palmCoA, incubated at 37 °C in acylation buffer, at various intervals. The sample was then immediately loaded into a capillary and separated.

The limits of detection for GAP-peptide and palm-GAP-peptide product were measured by CE. Increasing concentrations of GAP-

peptide and palm-GAP-peptide were sampled and individually electrophoresed. The limits of detection were defined as the lowest concentration detectable with a signal to noise ratio of three (S/N = 3).

The effect of pH on GAP-peptide palmitoylation was examined by mixing 10 μM GAP-peptide with 50 μM palmCoA in acylation buffers of varying pH, ranging from 7.0 to 11.4. After incubation for 10 min at 37 °C, the percent palmitoylation of the samples was determined by CE.

To examine the effect of 2-bromopalmitate (2-BP), a palmitoylation inhibitor, on GAP-peptide palmitoylation, 10 μM GAP-peptide and 50 μM palmCoA were mixed with 0–200 μM 2-BP and incubated for 10 min at 37 °C. The percentage of GAP-peptide palmitoylation in the presence and absence of 2-BP was plotted against concentration of 2-BP. The effect of pH on 2-BP inhibition was also investigated by mixing 10 μM GAP-peptide, 50 μM palmCoA, and 200 μM 2-BP in acylation buffers of varying pH. The percent palmitoylation of GAP-peptide was determined by CE following incubation for 10 min at 37 °C.

2.7. Correction of peptide fluorescence intensity changes

The difference in fluorescence intensity between GAP-peptide and palm-GAP-peptide was corrected using the relative peak areas for GAP-peptide and palm-GAP-peptide generated from the fluorescence spectra in the MEKC electrophoretic buffer. To calculate the peptide palmitoylation percentage, the peak area corresponding to the palmitoylated peptide was divided by the total area of the palmitoylated plus non-palmitoylated substrate. The difference in fluorescence intensity between GAP-peptide and palm-GAP-peptide was accounted for by multiplying the peak area of GAP-peptide by 1.54, the quotient of the palm-GAP-peptide peak area over GAP-peptide peak area from the fluorescence spectra in the MEKC electrophoretic buffer. The peak area for GAP-peptide was adjusted in this manner in all calculations of percentage palmitoylation.

2.8. Capillary electrophoresis with laser-induced fluorescence detection

CE with fluorescence detection was performed on a custom-built system with on-column detection. Briefly, a detection window was created by removing a portion (approximately 2 cm) of the polyimide coating 38 cm from the inlet of a 50 μm i.d. fused silica capillary tube (total length, 52 cm). A fiber optic cable with focusing optics (OZ Optics, Ottawa, ON, CA) was used to excite analytes passing through the window with 473 nm light from a diode-pumped solid state laser (BML-473-10F0A1FCA, Lasermate Group, Inc., Pomona, CA, USA). The light emitted perpendicular to the excitation beam was collected and focused using a microscope objective (40×, 0.75 n.a., Plan Fluor, Nikon, Melville, NY, USA) and filtered with a 473 nm notch filter (Razor Edge, Semrock, Rochester, NY, USA) and a 535/50 nm bandpass filter (Chroma Technology, Rockingham, VT, USA) before detection by a photomultiplier tube (PMT, R928, Hammamatsu, Bridgewater, CT, USA). The PMT current was amplified and converted to voltage with a preamplifier (model 1212, DL Instruments, Dryden, NY, USA) and digitized (DAS-1800, Keithly Metrabyte, Taunton, MA, USA) for readout on a personal computer.

The electrophoretic voltage was supplied by a high voltage power supply (CZE1000R, Spellman, Plainview, NY, USA). The electrophoretic buffer was 100 mM Tris base, 10 mM CTAB, and 10% ethanol, at pH 8.5. The inlet reservoir was held at ground potential and the outlet was held at a positive voltage of 15–20 kV. Between each run, the capillary was rinsed with 1 M NaOH for 2 min, followed by electrophoretic buffer for 2 min. Samples were loaded onto the capillary by gravitational fluid flow, with the loaded volumes calculated using Poiseulle's equation.

3. Results and discussion

3.1. Design and selection of substrate

A peptide substrate (GAP-peptide: HiLyteFluor488-MLCCMRRTK) derived from the N-terminal 9-amino acids of the growth-associated protein GAP-43 (also known as neuromodulin) was used as a substrate since this sequence is palmitoylated *in vivo* [22,39]. GAP-peptide possesses two cysteine residues, both of which are palmitoylated in the protein, thereby increasing the chances of observing a palmitoylation event in the assay. Additionally, the presence of a lysine and two arginine residues enhanced the solubility of this peptide sequence in aqueous buffers. Unlike other palmitoylated proteins, such as ras and many G proteins, which undergo additional lipid modifications [9,40], the GAP protein is only palmitoylated at its amino terminus. Accordingly, the separation of a basic, hydrophilic peptide from a hydrophobic, palmitoylated peptide should be accomplishable by CE. To track palmitoylation of the peptide, a fluorescein-based fluorophore, HiLyteFluor488, was used to label GAP-peptide. HiLyteFluor488 was chosen for its photostability and pH insensitivity [41], allowing sensitive detection of GAP-peptide and its palmitoylated product in this CE-based assay.

3.2. In vitro non-enzymatic palmitoylation of the GAP-peptide substrate

Two mechanisms have been proposed for the palmitoylation of peptides and proteins: non-enzymatic (spontaneous) palmitoylation and enzymatic palmitoylation. Non-enzymatic palmitoylation occurs in the presence of long-chain acylCoAs. Studies of numerous peptides *in vitro* [29,30,42] have shown that non-enzymatic palmitoylation is largely dependent on the formation of thiolate ions ($RS:^-$). Non-enzymatic palmitoylation occurs when conditions favor the formation of a thiolate ion, such as an increase in pH or the presence of basic and/or aromatic amino acid residues nearby the cysteine to be palmitoylated. Initial efforts to determine whether GAP-peptide could be palmitoylated utilized non-enzymatic palmitoylation. GAP-peptide was mixed with an excess of palmCoA in acylation buffer at 37 °C and incubated for 4 h. Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) was used to monitor the progress of the reaction and to confirm the formation of the palmitoylated product. The molecular weight of GAP-peptide alone, as determined by MALDI-MS (1499 m/z), corresponded to the theoretical molecular mass of the peptide plus one hydrogen atom (Fig. 1A). After four hours of incubation, a major peak with a molecular weight of 1976 m/z was present by MALDI-MS. This corresponds to the mass of GAP-peptide with the addition of two palmitoyl groups (Fig. 1B). The peaks located at 500–600 m/z and 1100 m/z correspond to the presence of buffer salts and excess palmitoyl coenzyme A, respectively, in the sample. A minor secondary product possessed the same molecular weight as that of GAP-peptide with a single palmitoyl moiety (1738 m/z). Thus the end product of GAP-peptide reaction with palmCoA was a doubly palmitoylated peptide (palm-GAP-peptide).

3.3. Fluorescence spectra of GAP-peptide substrate and palmitoylated GAP product

Previous work utilizing fluorescently-labeled peptide substrates for monitoring palmitoylation observed increases in fluorescence

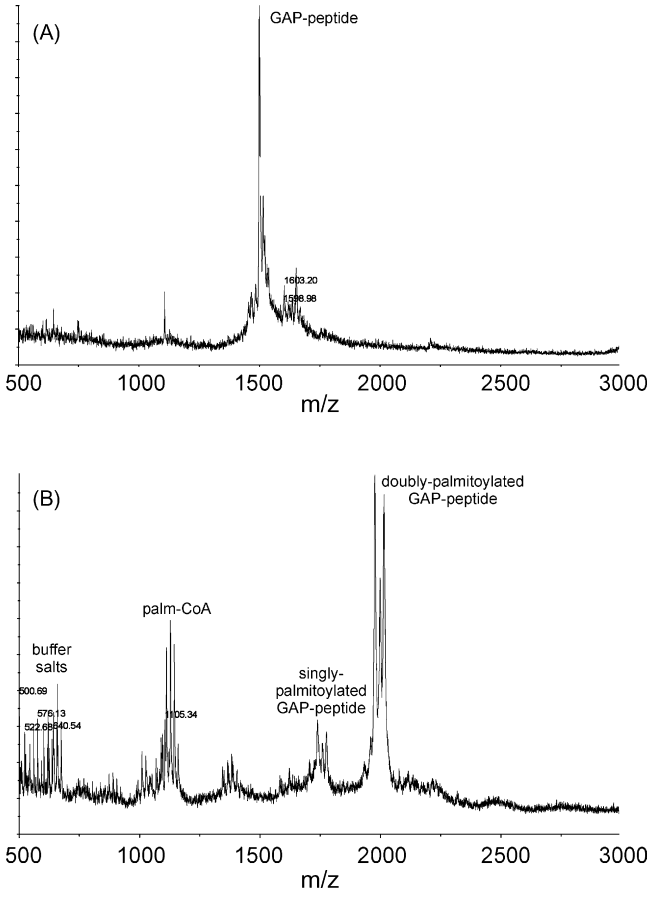

Fig. 1. MALDI-MS spectra of (A) the GAP-peptide substrate and (B) a mixture of GAP-peptide with an excess of palmitoyl coenzyme A after incubation at 37 °C for 4 h.

intensity following palmitoylation of a peptide [43]. To determine whether the fluorescence properties of GAP-peptide were altered by palmitoylation, the fluorescence spectra of the doubly palmitoylated and non-palmitoylated peptide were measured in a neutral aqueous buffer. When excited at 473 nm, the emission spectrum of GAP-peptide demonstrated a maximum at 526 nm (Fig. 2A). With the same excitation wavelength, the fluorescence emission maximum of the palm-GAP-peptide product was red shifted from that of GAP-peptide by 12 nm, to 538 nm (Fig. 2A). Since the fluorophore is in close proximity to the palmitoyl groups, the shift in the fluorescence emission of the palm-GAP-peptide product relative to that of GAP-peptide was most likely due to a change in the environment of the fluorophore created by the added palmitoyl groups. Interestingly, the maximal fluorescence intensity of palm-GAP-peptide was significantly smaller than that of GAP-peptide. The increase in the hydrophobicity of the environment near the fluorophore on the palm-GAP-peptide molecule may also explain the decrease in fluorescence intensity of the fluorophore. Palmitoyl moieties on palm-GAP-peptide may also increase aggregation of the palm-GAP-peptide molecules, inducing locally high concentrations of the fluorophores in the neutral aqueous buffer, thereby leading to fluorescence self-quenching. Self-quenching of the fluorescence of fluorophores such as BODIPY, NBD and DPH has been observed and exploited in assays probing the close interactions of peptides and phospholipids with membranes and vesicles [44–47].

The fluorescence properties of palm-GAP-peptide and GAP-peptide were also examined in a detergent-containing buffer, specifically the MEKC electrophoresis buffer used in the CE-based assay. The fluorescence of GAP-peptide, dissolved in a typical MEKC separation buffer containing CTAB, was red shifted with a peak emission of 528 nm while the peak emission of the palm-GAP-peptide product was blue shifted to 532 nm (Fig. 2B). The peak intensity of both GAP-peptide and palm-GAP-peptide increased in the MEKC buffer. While the peak intensity for GAP-peptide increased 1.5 times between the aqueous buffer and the hydrophobic, CTAB-containing buffer, the fluorescence peak intensity of the palm-GAP-peptide product in the CTAB buffer increased eight times. The significant increase in fluorescence intensity of the palm-GAP-peptide product, when dissolved in the hydrophobic buffer, again suggests that the fluorescence properties of the fluorophore on the peptide are environment sensitive [48–51]. Most importantly, the increase in fluorescence of palm-GAP-peptide in hydrophobic environments, such as those used in MEKC run buffers, should be considered when using fluorescence intensity to quantify the relative amounts of GAP-peptide to the palm-GAP-peptide product.

3.4. CE separation of palmitoylated GAP-peptide

To determine whether GAP-peptide and palmitoylated GAP-peptide could be separated by MEKC in a capillary, numerous electrophoretic buffers were tested. Previously, hydrophobic, farnesylated pentapeptides were successfully separated from hydrophilic peptide substrates using MEKC electrophoretic buffers containing tetraborate mixed with SDS or β-cyclodextrin [37]; however, the present study investigated the separation of basic, palmitoylated peptide substrates by MEKC. Tetraborate and Tris base buffers, mixed with differing concentrations of surfactants, were tested for separation efficiency and resolution of GAP-peptide and palm-GAP-peptide product. The negatively charged surfactants sodium dodecylsulfate (SDS) and sodium deoxycholate (SDC), the neutral surfactant Triton X, and the positively charged surfactant

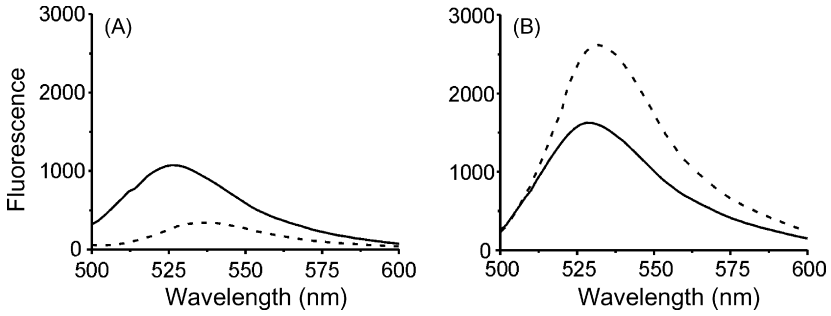

Fig. 2. Fluorescence spectra of GAP-peptide substrate and palmitoylated GAP-peptide product. GAP-peptide (solid line) and palm-GAP-peptide (dashed line) (1 μM each) were dissolved in (A) a neutral aqueous buffer and (B) an electrophoretic buffer containing CTAB and excited at 473 nm. Peak areas generated from the fluorescence spectra in (B) were used to correct the peak areas of the GAP-peptide and palm-GAP-peptide for their differing fluorescence intensities.

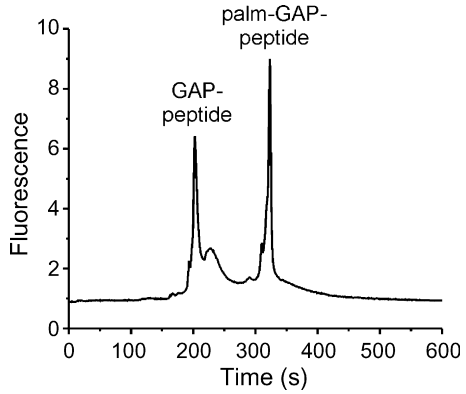

Fig. 3. Separation of GAP-peptide and palm-GAP-peptide in tetraborate (25 mM) and CTAB (10 mM). The capillary outlet was held at a positive (+22 kV) potential with the inlet at ground. A bare, 50 μm i.d., fused silica capillary was used.

cetyl trimethylammonium bromide (CTAB) were tested as MEKC additives.

Electrophoresis of GAP-peptide and palm-GAP-peptide in tetraborate with SDS yielded a single peak with no evidence of separation of the two analytes. While this electrophoretic buffer system was effective in separating farnesylated pentapeptides [37], tetraborate with SDS was ineffective in separating GAP-peptide from palm-GAP-peptide. Similar results were observed with electrophoretic buffers containing tetraborate with SDC, Tris with SDS, and Tris with SDC (data not shown). Another MEKC electrophoresis buffer, tetraborate with Triton X, produced two overlapping and broad peaks for GAP-peptide and palm-GAP-peptide, suggesting that adsorption to the capillary wall might be substantial. Repeated attempts to improve resolution with organic additives and optimization of the Triton X concentration failed to effectively separate the peaks. A Tris-based electrophoretic buffer containing Triton X was also ineffective in separating GAP-peptide from palm-GAP-peptide (data not shown). Only electrophoretic buffers containing CTAB effectively separated GAP-peptide and palm-GAP-peptide into two major peaks (Fig. 3). In these separations, GAP-peptide migrated first, followed by palm-GAP-peptide. Both Tris and tetraborate-based buffers containing CTAB were effective in separating GAP-peptide from palm-GAP-peptide (data not shown).

The effect of the organic modifiers urea, acetonitrile, and various alcohols, on the separation efficiency of the CTAB-containing electrophoresis buffers was also examined (data not shown). While most of the organic modifiers were successful in increasing resolution and efficiency of GAP-peptide and palm-GAP-peptide peaks in the CTAB-containing buffers, addition of 10% ethanol was most effective in improving separation and peak shape of both GAP-peptide and palm-GAP-peptide (Fig. 4A).

Of the buffers tested, only those containing the surfactant CTAB were capable of separating GAP-peptide from palm-GAP-peptide. The electrophoretic buffer used to separate GAP-peptide from palm-GAP-peptide in the following experiments was composed of 100 mM Tris, 10 mM CTAB, 10% ethanol, pH 8.5. Electrophoresis of a mixture of GAP-peptide and palm-GAP-peptide produced two well-separated peaks (Fig. 4A). Both GAP-peptide and palm-GAP-peptide were separated and baseline resolved in less than 7 min. The migration time was reproducible over nine consecutive runs in one day; however, the peak heights and areas showed more variation (Table 1). Resolution of the peaks was good, at 2.5. The efficiency of the separation ranged from 5200 plates (11,555 plates/m) for GAP-peptide to 37,000 plates (82,222 plates/m) for palm-GAP-peptide, which agrees well with previously reported values using MEKC to separate hydrophobic peptides [37].

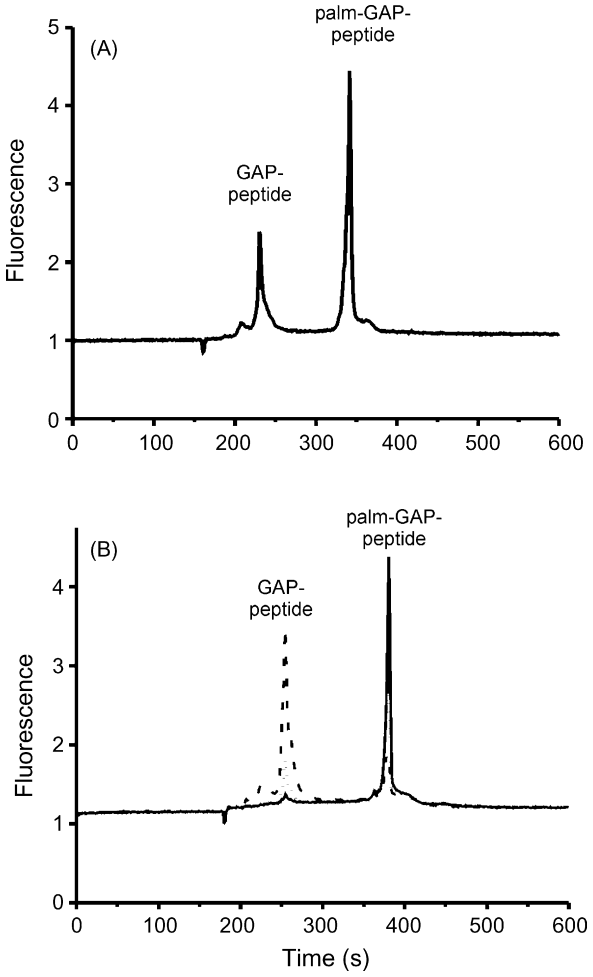

Fig. 4. (A) Separation of GAP-peptide and palm-GAP-peptide in a Tris/CTAB/EtOH electrophoresis buffer. (B) Separation of GAP-peptide (10 μM) following incubation with 100 μM palmitoyl coenzyme A for 10 min (long dash), 30 min (short dash), and 60 min (solid line) at 37 °C. Different capillaries were used for the separations in (A) and (B).

The GAP-peptide used in this study contains two arginine molecules and a single lysine residue at the carboxy terminal end, which imparts a strong positive charge to the molecule. The positively charged surfactant CTAB most likely acted as a dynamic coating for the capillary walls, preventing adsorption of the basic GAP-peptide to the negatively charged walls of the bare capillary. CTAB also functioned as a micellar pseudo-stationary phase, aiding the separation of GAP-peptide from the hydrophobic, palmitoylated GAP-peptide. Addition of ethanol to the CTAB-containing MEKC buffer changed the partitioning of GAP-peptide and palm-GAP-peptide into the aqueous phase from the micellar phase, which sharpened the peaks and improved the resolution.

The limits of detection for standards of GAP-peptide and palm-GAP-peptide were determined by loading decreasing concentrations of the peptides into a capillary and separating by CE. As the concentration of palm-GAP-peptide was decreased, the area of the

Table 1
% R.S.D. of the migration times, peak heights, peak areas and peak efficiencies (*N*) for GAP and GAP-palm from nine sequential runs

	Migration time	Peak height	Peak area	*N*
GAP-peptide	1.4	16.9	18.2	20.9
GAP-palm	2.5	13.9	12.6	53.9

fluorescence peak decreased linearly. Under these conditions, the detection limit for palm-GAP-peptide was 0.21 attomoles. A similar limit of detection for a farnesylated ras pentapeptide substrate was recently reported [37]. A detection limit of 3.0 attomoles was calculated for GAP-peptide. The differences between the detection limits of GAP-peptide and palm-GAP-peptide are most likely caused by differences in their fluorescence intensity combined with residual adsorption of the peptides on the capillary walls and reaction vessels.

3.5. Reaction kinetics of non-enzymatic palmitoylation

Unlike whole proteins, peptide substrates used to study palmitoylation dynamics are often non-enzymatically palmitoylated by the palmCoA substrate. Therefore, it is useful to characterize the role of non-enzymatic palmitoylation in overall peptide palmitoylation dynamics, especially prior to using peptides as enzymatic probes. To determine the time scale for non-enzymatic palmitoylation, GAP-peptide was mixed with palmCoA and the percentage of palm-GAP-peptide measured by CE at varying times (Fig. 4B). Two dominant peaks were present in the electropherogram with migration times equivalent to that of the GAP-peptide and palm-GAP-peptide. No peaks attributable to a singly palmitoylated peptide could be identified. At 20 min, 40% of the peptide was doubly palmitoylated and by 60 min, 90% of the peptide was labeled with two palmitoyl groups (Fig. 5A).

During this study of non-enzymatic palmitoylation, no electrophoretic evidence corresponding to the presence of a singly palmitoylated GAP-peptide was observed in contrast to the results of the MALDI-MS. There are two possible explanations for these differing results. First, significant quantities of singly palmitoylated GAP-peptide may never have formed in the reaction, due to rapid

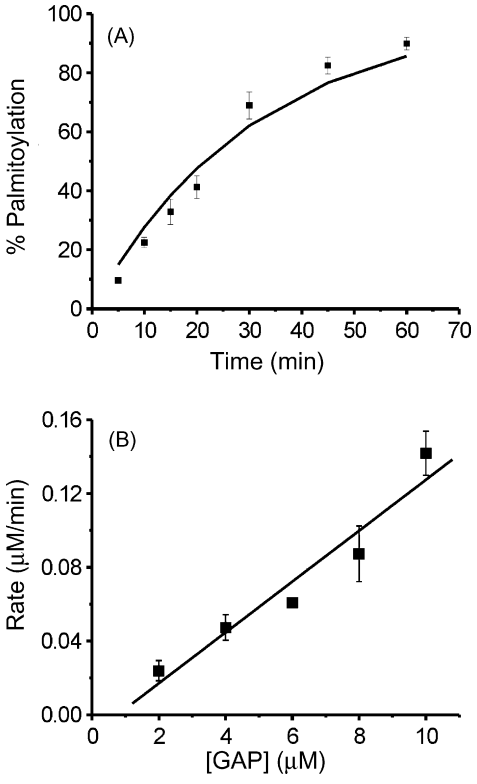

Fig. 5. Time dependence and rate of GAP-peptide palmitoylation. (A) Time dependence of GAP-peptide palmitoylation. Each point represents the % palmitoylation ± standard error ($n = 5$). (B) Dependence of GAP-peptide palmitoylation on the GAP-peptide concentration. Each point is an average ± S.D. ($n = 3$).

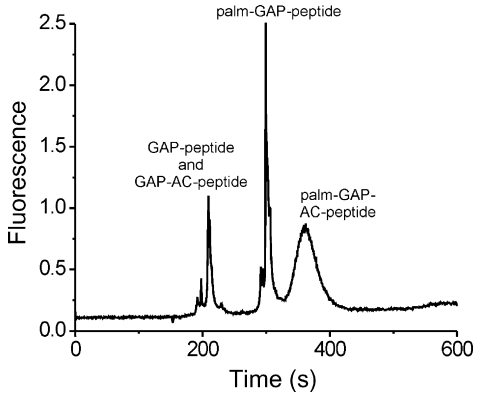

Fig. 6. Separation of a mixture of GAP-peptide, GAP-AC-peptide, palm-GAP-peptide, and palm-GAP-AC-peptide in a Tris/CTAB/EtOH electrophoresis buffer.

conversion of the singly palmitoylated GAP-peptide to the doubly palmitoylated palm-GAP-peptide. At no time point during the reaction was a peak attributable to the singly palmitoylated GAP-peptide observed. While there is evidence of singly palmitoylated GAP-peptide in the MALDI-MS data of a mixture of GAP-peptide and palmCoA incubated to reaction completion (>4 h at 37 °C) (Fig. 1B), these data may be misleading. Laser desorption of the palmitoylation reaction mixture in MALDI-MS may have resulted in cleavage of a single palmitoyl group from doubly palmitoylated palm-GAP-peptide molecules, resulting in the appearance of singly palmitoylated GAP-peptide molecules.

It is, however, conceivable that singly palmitoylated GAP-peptide is not observed during electrophoretic measurements of GAP-peptide palmitoylation because singly and doubly palmitoylated GAP-peptides co-migrate. However, an exhaustive search of over a dozen different electrophoresis buffers was performed to separate the constituents of the GAP-peptide palmitoylation reaction mixtures at numerous time points. At no time point during the palmitoylation reaction was a peak with a migration time indicative of a third species (singly palmitoylated peptide) consistently present on the electropherograms. Given these results, experiments were designed to investigate where singly palmitoylated GAP-peptide might migrate in a CTAB-based electrophoretic buffer.

To determine where a singly palmitoylated peptide would migrate under the chosen electrophoretic conditions, a version of GAP-peptide with one cysteine replaced by an alanine was synthesized (GAP-AC-peptide). Although the separation conditions were not optimized for the separation of palm-GAP-AC-peptide from palm-GAP-peptide, electrophoresis of a mixture of GAP-peptide, GAP-AC-peptide, palm-GAP-peptide and palm-GAP-AC-peptide produced three resolved peaks (Fig. 6). GAP-peptide and GAP-AC-peptide co-migrated first, followed by palm-GAP-peptide and palm-GAP-AC-peptide, which were separated and baseline resolved. These data suggest that the peak observed following non-enzymatic palmitoylation of GAP-peptide by palmCoA consists solely of doubly palmitoylated GAP-peptide.

In an attempt to promote, separate, and detect the formation of singly palmitoylated GAP-peptide, the effect of palmCoA concentration on the rate of GAP-peptide palmitoylation was examined. Samples containing varying concentrations (10–50 μM) of palmCoA were incubated with GAP-peptide and the initial velocity of the reaction was measured. Between 10 and 50 μM palmCoA, the rate of palmitoylation of GAP-peptide was independent of the concentration of palmCoA (data not shown). This observation agrees with prior investigations of palmitoylation of peptides based on the ras protein [29]. Furthermore, when palmCoA concentrations were

lowered to levels below the CMC for palmCoA [52], eliminating hydrophobic interactions which might promote rapid conversion of singly palmitoylated GAP-peptide to doubly palmitoylated GAP-peptide, only two peaks, with migration times corresponding to GAP-peptide and doubly palmitoylated GAP-peptide, were observed. These data also suggest that lowering palmCoA concentration below the CMC does not promote the formation of singly palmitoylated GAP-peptide.

3.6. Dependence of palmitoylation on GAP-peptide concentration

To determine the effect of GAP-peptide concentration on the rate of non-enzymatic peptide palmitoylation, the reaction rate was measured as the concentration of GAP-peptide was varied. The initial velocity of the reaction was determined by measuring the amount of palm-GAP-peptide product before 15% of the GAP-peptide substrate was consumed. Again in these experiments no singly palmitoylated peptide was observed. The rate of GAP-peptide palmitoylation was proportional to the concentration of the GAP-peptide (Fig. 5B). This observation agrees with previous reports [9,42,53,54] suggesting that formation of the thiolate ion determines the rate of palmitoylation of the peptide. The formation of the thiolate ion is likely determined by the environment of the cysteine side chain on the GAP-peptide molecule, *i.e.* the presence of basic residues (arginine and lysine), and perhaps even the presence of a neighboring cysteine.

3.7. Dependence of palmitoylation on pH

Since pH influences thiolate formation, the effect of pH on GAP-peptide palmitoylation was examined. The amount of palmitoylated peptide was measured after incubation of GAP-peptide with palmCoA at varying pH between 7.0 and 11.4. Above pH 7.0, nearly all of the GAP-peptide molecules were doubly palmitoylated (Fig. 7A). This substantial increase in the total amount of palmitoylation occurring in the assay at this time was likely due to thiolate ion formation on the cysteine side chain. As the pH of the reaction approached the pK_a of the thiol groups of the cysteines in the GAP-peptide, thiolate formation increased, resulting in increased palmitoylation. While experimental determination of the pK_a values of the two cysteines on the GAP-peptide is feasible [42], it was beyond the scope of this study. Nonetheless, increasing the pH of the palmitoylation reaction buffer above 7.0 significantly increased GAP-peptide palmitoylation. Since an increase in pH significantly increases the rate of non-enzymatic palmitoylation, pH conditions must be chosen carefully to limit the contribution from non-enzymatic palmitoylation when investigating enzymatic palmitoylation.

3.8. Inhibition of the reaction by 2-bromopalmitate (2-BP)

2-Bromopalmitate is a commonly used inhibitor for enzymatically catalyzed palmitoylation; however, its use to block non-enzymatic palmitoylation has not been reported. The ability of 2-BP to inhibit palmitoylation of GAP-peptide mediated by palmCoA alone was measured. 2-BP, at concentrations known to inhibit enzymatic peptide palmitoylation [30,43], inhibited non-enzymatic GAP-peptide palmitoylation in a concentration-dependent manner (Fig. 7B). Previous studies suggested that 2-BP formed an irreversible covalent bond with acyltransferases [55–58], blocking enzymatic palmitoylation of downstream molecules, such as carnitine and proteins. These data suggest that 2-BP can also block the palmitoylation reaction by a mechanism other than direct reaction with acyltransferase, the enzyme responsible for palmitoylation *in vivo*.

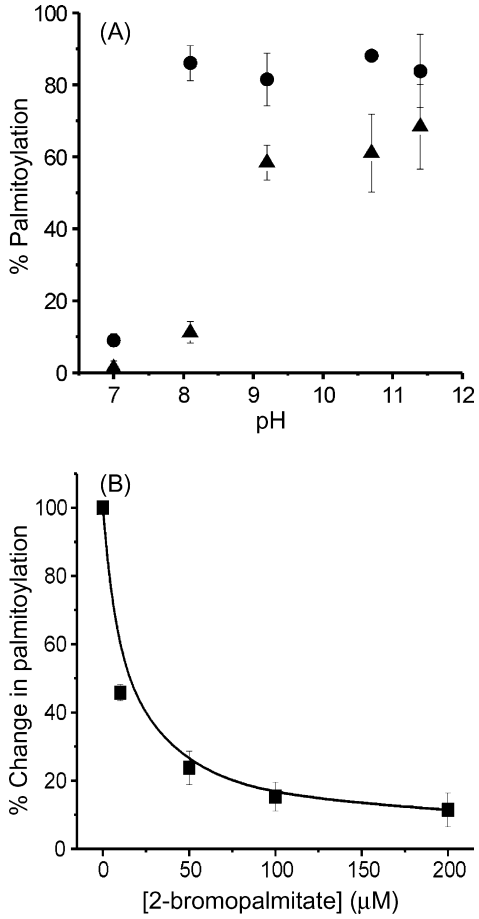

Fig. 7. Factors affecting non-enzymatic GAP-peptide palmitoylation. (A) Dependence of GAP-peptide palmitoylation on pH (solid circles) and the effect of increasing pH on GAP-peptide palmitoylation in the presence of 200 μM 2-BP (solid triangles). (B) Inhibition of palmitoylation by 2-bromopalmitate (2-BP). Each point is an average ± standard deviation of 3 trials.

To gain insight into the mechanism whereby 2-BP blocked palmitoylation by palmCoA, 2-BP was incubated with GAP-peptide and palmCoA in varying pH solutions. Even in the presence of 2-BP at four times the concentration required to inhibit enzymatic palmitoylation, GAP-peptide palmitoylation increased as pH was increased (Fig. 7C). An increase in pH could overcome the inhibitory effect of 2-BP, suggesting that 2-BP may inhibit palmitoylation by blocking thiolate formation at neutral pH.

4. Conclusions

This report described the development of a CE-based assay for peptide palmitoylation. Non-enzymatic palmitoylation of a small peptide substrate was quantified *in vitro*, including examination of palmitoylation with respect to reaction time, GAP-peptide concentration, pH, and inhibitor concentration. In the future, this CE-based assay for palmitoylation may be used to measure and monitor peptide or protein de-palmitoylation, which cannot be quantitatively measured by current radioisotope labeling techniques. This separation method, based on the differing hydrophobicities of the non-palmitoylated and palmitoylated peptides, may be applicable to other palmitoylated peptides or small proteins; however, the structure of the peptide or protein plays a large role in the efficiency of separation, especially in bare capillaries. Future work will address peptide–capillary wall interactions, which decreases efficiency and increases the limit of detection. Neutral, hydrophilic, covalent

capillary coatings may be the key to improving both sensitivity and selectivity by preventing substrate loss to the capillary walls.

The techniques and methods reported above may be used to measure the activity of acyltransferases and thioesterases, the enzymes responsible for adding and removing palmitate moieties to peptides and proteins inside living cells. These enzymes, through their actions to palmitoylate and de-palmitoylate peptides and proteins, play an integral role in the regulation of protein trafficking, protein–protein interactions, as well as enzyme activity. The combined use of fluorescently-labeled peptide substrates and CE will enable the monitoring, both visually and quantitatively, of substrates inside living cells as a function of enzymatic palmitoylation. Because this CE-based method uses very small volumes and can detect very low concentrations of fluorescently-labeled substrate, future studies will focus on the measurement of palmitoylation dynamics in single cells.

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