Pseudo-enzymatic S-acylation of a myristoylated Yes protein tyrosine kinase peptide *in vitro* may reflect non-enzymatic S-acylation *in vivo*

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Covalent attachment of a variety of lipid groups to proteins is now recognized as a major group of post-translational modifications. S-acylation of proteins at cysteine residues is the only modification considered dynamic and thus has the potential for regulating protein function and/or localization. The activities that catalyse reversible S-acylation have not been well characterized and it is not clear whether both the acylation and the deacylation steps are regulated, since in principle it would be sufficient to control only one of them. Both apparently enzymatic and non-enzymatic S-acylation of proteins have previously been reported. Here we show that a synthetic myristoylated c-Yes protein tyrosine kinase undecapeptide undergoes spontaneous S-

acylation *in vitro* when using a long chain acyl-CoA as acyl donor in the absence of any protein. The S-acylation was dependent on myristoylation of the substrate, the length of the incubation period, temperature and substrate concentration. When COS cell fractions were added to the S-acylation reaction no additional peptide:S-acyltransferase activity was detected. These results are consistent with the possibility that membrane-associated proteins may undergo S-acylation *in vivo* by non-enzymatic transfer of acyl groups from acyl-CoA. In this case, the S-acylation–deacylation process could be controlled by a regulated depalmitoylation mechanism.

INTRODUCTION

A large number of viral and cellular proteins have been found to be modified by fatty acylation [1–3]. Eukaryotic proteins can be modified by fatty acids in two ways. Fatty acids, usually the C₁₄ myristate, are added cotranslationally to an N-terminal glycine residue in amide linkage which is inherently stable under physiological conditions. This is generally thought to be irreversible *in vivo*, although recently an activity capable of specifically removing myristate was identified [4]. Acylation of proteins with longer chain fatty acids, most frequently the C₁₆ saturated fatty acid palmitate, is a post-translational modification. The fatty acid is attached via thioester linkage to one or more cysteine residues within the same protein that lie topologically close to the cytoplasmic face of cellular membranes [5]. This process has frequently been called palmitoylation, but is more properly termed S-acylation to take into account the attachment of a range of fatty acyl chain lengths of differing saturation [6–7]. S-acylation can be a reversible process [8] and its function is starting to be elucidated. It has been reported to affect signal transduction, receptor modulation, protein localization and lateral diffusion in cell membranes [9–12].

S-acylation and deacylation of proteins can be regulated by extracellular signals providing a mechanism for the dynamic regulation of protein localization [13]. Several heterotrimeric G-protein α -subunits and members of the Src family of protein tyrosine kinases (PTKs) are S-acylated, usually in a myristoylation-dependent manner [14–18]. Unlike other lipid modifications of proteins, such as myristoylation, prenylation and addition of glycosylphosphatidylinositol anchors, S-acyl groups often turn over with a much shorter half-life than that of the protein. This rapid turnover and the variation of S-acylation

levels with extracellular stimuli strongly suggest that addition or removal of fatty acid regulates the activity of proteins [13].

Although many studies have suggested the intracellular presence of enzymes catalysing the S-acylation of proteins, their identity has not been verified. So far, attempts to purify the protein:S-acyltransferases to homogeneity have proved difficult and the investigations have been limited to studies in crude or partially purified systems [19–27]. A thioesterase active on Ras and G-protein α subunits has recently been purified [28]. However, subsequent cloning of the cDNA encoding that enzyme showed it to be a secreted protein and therefore not the authentic deacylating enzyme for cytoplasmic proteins [29]. It has been suggested that transfer of S-acyl groups from acyl-CoA to rhodopsin [30] or the major proteolipid (P₀) of myelin [31–33] may be non-enzymatic. Lipid-modified cysteine-containing synthetic peptides were found to be efficiently S-acylated *in vitro* in the absence of any enzyme when presented with acyl-CoA in liposomes, apparently by a strictly chemical mechanism [34]. Acylation of UDP-glucuronosyl transferase from rat liver or acylation of G-protein α subunits occurred in the absence of any other protein, suggesting autoacylation [35,36]. On the other hand, a recent report [37] describes how a variety of cysteine-containing lipid modified peptides are found to be S-acylated by cultured mammalian cells. In this work the authors suggest that the process appears to be enzymatic.

In the present study we have examined the possibility that myristoylated peptides with cysteinyl residues may constitute efficient substrates for S-acylation. A simple Cys-containing myristoyl peptide, with a sequence modelled on the naturally occurring S-acylated protein c-Yes (a Src PTK family member), undergoes spontaneous S-acylation in the presence of [³H]-palmitoyl-CoA as acyl donor under physiological conditions.

Abbreviations used: ACBP, acyl CoA binding protein; CoA, coenzyme A; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; DTT, dithiothreitol; HPTLC, high performance thin layer chromatography; Pal-CoA, palmitoyl coenzyme A; PTK, protein tyrosine kinase; SUV, small unilamellar vesicles.

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This observation may have significant implications for understanding the mechanism of S-acylation *in vivo*.

EXPERIMENTAL PROCEDURES

Materials

Coenzyme A (CoA), actin, and phospholipids dioleoylphosphatidylcholine (DOPC), dioleoylphosphatidylethanolamine (DOPE), and dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma-Aldrich Co. (Dorset, U.K.). S-palmitoyl-coenzyme A (Pal-CoA) was from Boehringer-Mannheim U.K. Solvents were all spectroscopic grade and from Romil, Cambridge, U.K. Trifluoroacetic acid was from Pierce. [9,10-³H]-Palmitic acid and [9,10-³H]myristic acid (specific activity for both of them 40–60 Ci/mmol) were purchased from Amersham International (Amersham, U.K.). [³H]acetyl-CoA (specific activity 200 mCi/mmol) was from NEN-DuPont Ltd. (U.K.). Silica gel high performance thin layer chromatography (HPTLC) plates were from Merck (Darmstadt, Germany). Scintillation fluid was from Beckman Instruments (U.S.A.). Acyl-CoA binding protein (ACBP) was a kind gift of Dr. Jens Knudsen, Aarhus University, Denmark.

Peptide synthesis

The peptides used (Table 1) were synthesized by the NIMR Peptide Synthesis Service using standard Fmoc chemistry. The sequences were chosen as they have only one cysteine and several basic residues that confer a positive charge on the peptides and facilitate binding to negatively charged filters. Myristoylation of the N-terminal glycine was performed before the peptide was cleaved from the resin. Variants that have cysteine substituted by alanine at position three were used as a control for non-specific acylation not occurring at cysteine. All the peptides were purified by HPLC on a reverse phase Prep-10 Butyl Aquapore column (Applied Biosystems) using a 5–90% acetonitrile gradient with 0.1% trifluoroacetic acid. For analytical separations a 7 μ m Butyl Aquapore column was used. The purity and molecular mass of each peptide was determined by electrospray mass spectrometry (Micromass Platform mass spectrometer) with the help of Dr. S. Howell.

S-acylation assay

[³H]Palmitoyl-CoA ([³H]-Pal-CoA) was prepared according to the method previously described [38], and purified by loading onto a Sep-Pak C-18 cartridge (Waters) equilibrated with 50 mM ammonium acetate pH 5.3 and then eluting with 4 ml volumes of ammonium acetate-buffered 10, 20 and 60% acetonitrile. The

[³H]Pal-CoA eluted in the 60% acetonitrile fraction as confirmed by HPTLC using silica gel plates with a solvent system: chloroform/methanol/water, 4:3:1. The samples were concentrated by lyophilization to approximately the same radioactive concentration for each preparation and stored in aliquots at –70 °C.

The acylation reaction (final volume = 150 μ l) was carried out by incubating each peptide (at 10 or 100 μ M final concentration) at 37 °C with [³H]Pal-CoA (0.05–0.1 μ M final concentration) in acylation buffer [50 mM Hepes pH 7, 1 mM EGTA, 1 mM MgCl₂, 10 mM NaCl, 130 mM KCl and 1 mM dithiothreitol (DTT)]. DTT was included to maintain the reduced state of the sulphhydryl groups; it does not cleave S-acyl groups under these conditions [39]. Incubation time was 10 min unless otherwise stated. Microsomes or soluble fraction from COS cells or rat brain were added in some experiments. Aliquots (50 μ l) of the reaction mixture were spotted in duplicate on to phosphocellulose filters (Whatman P-81) [40], and the filters were then washed twice in 50 mM Hepes, pH 7 at 37 °C for 20 min each, once in methanol, dried and counted. [³H]Pal-CoA degradation was measured by partitioning of released [³H]palmitate into ethyl acetate and was found to be less than 15% after 30 min in all cases.

Preparation of vesicles

Small unilamellar lipid vesicles (SUV) were prepared from lipid mixtures in chloroform/methanol, 2:1; the solvent was evaporated under a nitrogen stream and the lipids hydrated by vortexing for 10 min at room temperature. The opaque suspension of multilamellar vesicles was next sonicated for 5 min on ice with a 0.5 cm flat disrupter tip mounted on a Soniprep 450 (MSE) sonifier, strength of pulse 4.5. After sonication, the vesicle suspension was centrifuged for 15 min at 35000 *g* to remove probe particles.

Subcellular fractionation and preparation of microsomal membranes

Microsomal membranes from COS cells were prepared as follows. COS cells were grown in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) newborn calf serum. When confluent, the cells were washed three times with ice cold PBS, scraped from the dishes and collected by low speed centrifugation at 4 °C. They were swelled by incubation in hypotonic buffer (5 mM Tris/HCl pH 7.5, 0.1 mM EDTA, 1 mM MgCl₂, containing aprotinin, leupeptin and pepstatin at 1 μ g/ml each and PMSF at 1 mM) on ice for 20 min. The cells were broken by passing the cell suspension through a narrow gauge needle several times, and unbroken cell debris and nuclei were removed by low speed centrifugation. The supernatant was then centrifuged at 100000 *g* for 30 min at 4 °C in a TL100.1-A rotor of a Beckman TL100 centrifuge. The soluble fraction (S-100) was used immediately or stored at –70 °C. The pelleted membrane fraction (P-100) was resuspended in buffer, recentrifuged and then resuspended in buffer before being stored at –70 °C. Microsomal membranes from rat brain were prepared similarly. In short, brains were removed from the animals, rinsed briefly in PBS, and chopped roughly with a blade in 5 mM Hepes pH 7.2, 1 mM EGTA, 1 mM DTT, 1 mM MgCl₂, 1% aprotinin, 0.1 mM EDTA. The tissue was broken up using a Polytron homogenizer and a glass Dounce homogenizer and then centrifuged at 3000 rev./min for 10 min. The supernatant was collected and centrifuged at 100000 *g* as for the COS cells. Protein concen-

Table 1 Synthetic peptide substrates corresponding to the amino terminal sequence of the Src family PTK, c-Yes

The myristoylated peptides were prepared as the C-terminal amide. Numbering corresponds to the primary translation product; the single letter amino acid code is used.

Peptide	Sequence
Yes-A ₃	GAIKSKEDKG
Myr-Yes-A ₃	Myr-GAIKSKEDKGH
Yes-C ₃	GCIKSKEDKG
Myr-Yes-C ₃	Myr-GCIKSKEDKGH

tration was measured with the Bradford method (Pierce and Warriner, Chester, U.K.).

SDS/polyacrylamide gel electrophoresis and fluorography

Labelled proteins were analysed by electrophoresis as previously described [18] and were stained with Coomassie Brilliant Blue R-250 or incubated with DMSO and then soaked in 2,5-diphenyloxazole/DMSO for fluorography [39]. The gels were dried and exposed to a pre-flashed XAR 5 autoradiographic film (Kodak) for at least 2 weeks at -70°C .

Figure 1 Isolation and characterization of the S-acylation product

(A) HPLC elution profile of Myr-Yes- C_3 peptide; the acetonitrile (ACN) concentration is indicated on the right and the absorbance at 230 nm is indicated on the left. The two peaks arrowed correspond to the peptide with the oxidized (o) and reduced (r) sulphhydryl group on the Cys. (B) HPLC elution profile after 30 min reaction at 37°C of Myr-Yes- C_3 peptide with Pal-CoA ($20\text{ }\mu\text{M}$ each). The new peak (arrowed, p) was identified as palmitoylated Myr-Yes- C_3 by electrospray mass spectrometry (C). A major A series corresponding to the expected product was observed as well as a minor B series corresponding to an unidentified product present at less than half of the abundance of the authentic product. The peak at m/z 460 is due to a contaminant commonly observed in a variety of samples, probably a plasticiser from plastic tubes.

RESULTS

Characterization of the chemical acylation of peptides

Cysteine-containing synthetic peptides were found by electrospray mass spectrometry to contain a roughly equal mixture of reduced and oxidized sulphhydryl groups, the latter eluting slightly ahead of the former (data not shown). Figure 1(A) shows the HPLC elution profile of Myr-Yes- C_3 (Table 1). Two peaks were observed corresponding to these two forms. After incubation of Myr-Yes- C_3 with unlabelled Pal-CoA (both at $20\text{ }\mu\text{M}$ final concentrations) at 37°C for 30 min in acylation buffer, aliquots of the reaction were analysed by HPLC. Figure 1(B) shows the elution profile of the reaction mixture. The second peak of Myr-Yes- C_3 corresponding to the reduced form was largely absent and a new peak appeared late in the acetonitrile gradient indicating almost quantitative conversion to a product with higher hydrophobicity than the original substrate. It was collected and directly analysed by electrospray mass spectrometry (Figure 1C). The product gave a major A series of peaks corresponding to a molecular mass of 1649.4 ± 0.14 , consistent with the product being palmitoylated Myr-Yes- C_3 . Primarily the triply (A3) and quadruply (A4) charged species were observed due to the analysis being performed under acid conditions where the three lysine and one histidine residues are largely protonated. A minor B series was also observed in which the doubly charged B2 peak predominated. The identity of this compound is unknown but it may represent a minor form of the peptide in which one of the lysine residues has become modified. The early eluting peak of Myr-Yes- C_3 representing the oxidized form was unchanged. No new products were detected when the other three peptides (Table 1) were used under similar conditions (data not shown). These results suggested that the palmitate was thioesterified to the cysteine residue because the oxidized peptide did not react and there was no acylation when Cys was substituted by Ala. The data also show that the myristoylation of the N-terminal glycine is necessary for the S-acylation reaction because no acylation was detected with non-myristoylated peptide (not shown).

To attempt to develop a more rapid and sensitive assay, we tried to bind the basic Yes peptides to anionic phosphocellulose paper filters. ^3H -labelled Pal-CoA was synthesized (see Experimental procedures) and the S-acylation reaction was carried out under the same conditions as above, except that the Myr-Yes peptide concentration was 10 or $100\text{ }\mu\text{M}$ and the ^3H Pal-CoA concentration was $0.05\text{ }\mu\text{M}$. Control experiments demonstrated that ^3H Pal-CoA was not retained on the filters (data not shown) and blank reactions without peptide were routinely run to correct for this low background. The four peptides were reacted and allowed to bind to phosphocellulose filters which were then washed and counted. As shown in Table 2(A), radioactivity was only found to be retained on the filters when Myr-Yes- C_3 was used. Treatment with 1 M hydroxylamine at pH 7 prior to filter binding removed essentially all the radioactivity associated with the peptide, indicating that the fatty acid is linked via a thioester bond. No effect was found when the samples were incubated with 1 M Tris/HCl buffer as a control (data not shown).

To investigate possible factors that could affect the S-acylation reaction, the palmitoylation of the peptides was carried out under different experimental conditions. Figure 2(A) shows the pH-dependence of the reaction. A steady increase in peptide acylation is detected when increasing the pH from 6 to 8. At pH 7 the reaction was approximately half as efficient as at pH 8. pH 7 was chosen for further experiments because this pH minimizes chemical acylation and thus is more suitable for detecting any possible enzyme activity. A time course of the S-acylation reaction is shown in Figure 2(B). When Myr-Yes- C_3

Table 2 Properties of the *in vitro* S-acylation assay

S-acylation activity (fmol of labelled fatty acid) was calculated from ^3H dpm retained on the phosphocellulose filters. Mean values are given $\pm$ the SEM. Values are derived from at least three independent experiments. **A** The peptides indicated (100 μM each) were incubated with 0.05 μM [^3H]Pal-CoA. **B** Cos cell microsomes were added in the indicated amounts of protein to reaction mixtures containing 100 μM Myr-Yes-C₃ and 0.1 μM [^3H]Pal-CoA. **C** The indicated amounts of Cos membrane or soluble proteins were added to reaction containing 100 μM Myr-Yes-C₃ and 0.1 μM [^3H]Pal CoA or [^3H]Myr-CoA. **D** Myr-Yes-C₃ (100 μM) and [^3H]Pal-CoA (0.05 μM) were reacted in the presence or absence of Cos cell microsomes, with or without 0.1% Triton X-100. **E** Myr-Yes-C₃ (100 μM) and [^3H]Pal-CoA (0.05 μM) were reacted in the presence or absence of Cos cell microsomes, with increasing concentrations of SUV liposomes. **F** Myr-Yes-C₃ (100 μM) and [^3H]Pal-CoA (0.05 μM) were reacted in the presence of the indicated amounts of ACBP.

		S-acylation activity (fmol)							
A Peptide ...		Yes-A3		Yes-C3		Myr-Yes-A3		Myr-Yes-C3	
		3.6 $\pm$ 26.1		0 $\pm$ 18.9		0 $\pm$ 13.9		173.2 $\pm$ 32.8	
B Cos membrane protein (μg) ...	0			0.76		1.5		3.8	
		275.4 $\pm$ 8.1		215.1 $\pm$ 36.0		171.0 $\pm$ 36.0		121.0 $\pm$ 30.1	
C Cos protein (μg) ...						1.5 (membrane)		1.5 (soluble)	
	CoA derivative ...	Pal		Myr		Pal		Myr	
		290.7 $\pm$ 5.4		228.6 $\pm$ 55.3		228.6 $\pm$ 20.7		121.0 $\pm$ 24.3	
								207.9 $\pm$ 17.1	235.3 $\pm$ 51.7
D Cos membrane protein (μg) ...	0					3			
	Triton X-100 ...	—		+		—		+	
		228.6 $\pm$ 24.3		405.0 $\pm$ 17.1		207.9 $\pm$ 46.8		155.7 $\pm$ 41.4	
E Liposome concentration (mM) ...	0			1		5		10	
								15	
Cos membrane protein (μg) ...	0	3		0	3	0	3	0	3
		306.0 $\pm$ 2.0	151.6 $\pm$ 38.7	270.0 $\pm$ 22.0	82.8 $\pm$ 27.4	171.0 $\pm$ 27.4	88.6 $\pm$ 24.7	76.0 $\pm$ 16.6	60.7 $\pm$ 22.0
								88.2 $\pm$ 6.6	24.75 $\pm$ 12.0
F ACBP conc. (μM) ...	0			10		20		50	
								100	
		733.9 $\pm$ 34.6		574.6 $\pm$ 27.9		373.9 $\pm$ 24.7		307.3 $\pm$ 41.4	297.9 $\pm$ 13.9

was incubated with [^3H]Pal-CoA, the label was incorporated into the peptide in a time-dependent manner. Following this result, the incubation time was reduced in some experiments to 10 min to be in the linear range of the assay. Figure 2(B) also shows that only background levels of counts were incorporated at 0 °C even over 60 min. The temperature dependence for the S-acylation reaction was also studied and the optimum temperature was found to be between 37 and 50 °C (data not shown), but significant reaction (80 % and 40 % of the 37 °C values) still occurred at 70 °C and 100 °C, respectively. We chose to run further reactions at 37 °C in order to approximate physiological conditions.

The dependency of S-acylation on the concentrations of both substrates [^3H]Pal-CoA and Myr-Yes-C₃ peptide is shown in Figure 3. Using a fixed peptide concentration (10 μM) and increasing the amount of labelled Pal-CoA up to 0.15 μM , the reaction is linear but saturation is not reached (Figure 3A). It was not possible further to increase the [^3H]Pal-CoA concentration for technical reasons. After 10 min of incubation with [^3H]Pal-CoA, the reaction shows a hyperbolic curve when varying the concentration of the peptide (Figure 3B). Transformation of the data using the Eadie-Hofstee method (Figure 4B insert) allowed the calculation of pseudo $K_m = 0.35 \mu\text{M}$ and $V_{\max} = 8841 \text{ dpm} \cdot \mu\text{g}^{-1} \cdot \text{min}^{-1}$ values, in good agreement with previous results with a different peptide substrate [33].

Critical micellar concentration (CMC) of Myr-Yes peptide

In order to understand why the S-acylation reaction occurs only when the peptide is myristoylated, we investigated whether Myr-

Yes-C₃ was able to form aggregated structures like micelles. The critical micellar concentration was calculated by two different methods previously described [41,42], both giving a CMC value between 50 and 100 μM (data not shown). Much of the data presented here were collected at 10 μM peptide, well below the CMC. If micelles are necessary for the reaction to occur, then when peptide concentrations are higher than 50 μM , a large increase in the product would be detected with little reaction below this concentration. The results in Figure 3(B) do not show such a discontinuity, suggesting that peptide micelles are not essential for the acylation. The concentration of Pal-CoA in the assay is 0.05 μM , well below the published CMC of 42 μM [43].

Acylation of the peptides with different subcellular fractions

Having characterized the chemical acylation, we attempted to apply the same experimental protocol to detect any possible enzymatic activity that may catalyse the transfer of the palmitoyl group from Pal-CoA to the peptides. For this purpose, different amounts of a COS cell microsomal fraction (P-100) were added to the reaction to detect an increase in palmitoylated peptide if an acyltransferase enzyme was present. Instead of an increase, a decrease in the amount of radioactive product was found in all cases (Table 2B). The COS cell soluble fraction (S-100) was also assayed, but again a slight decrease was detected (data not shown). To be sure that the effect was not due to the kind of cells used, a rat brain microsomal preparation was also tested but with similar results (data not shown). Prior boiling of the

Figure 2 pH dependence (A) and time course (B) of the S-acylation reaction with Myr-Yes-C₃

(A) 50 mM MES buffer was used from pH 5 to pH 7 (○) and 50 mM Hepes buffer from pH 6.5 to 8 (●). (B) Samples were incubated at 0 °C (○) or 37 °C (●) and aliquots were counted at different times using the phosphocellulose filter binding assay. The peptide concentration was 100 μM. Values are derived from three independent experiments.

microsomes or treatment with proteinase K did not substantially affect the results. In fact, the decrease in the amount of labelled peptide was less than without any treatment (data not shown), suggesting that a deacylase acting on the acylated peptide may be partially responsible for the observed decrease. As noted in the Experimental section, less than 15% of the acyl-CoA was degraded under these conditions.

To determine the acyl group specificity three different substrates were used, [³H]Pal-CoA, [³H]myristoyl-CoA and [³H]acetyl-CoA. Acylation of Myr-Yes-C₃ was efficient with both the long chain fatty acyl CoAs, indicating that the acylation reaction has a broad specificity for fatty acyl CoA (Table 2C). No acetylated product was detected when using [³H]acetyl-CoA. Palmitate, either alone or with ATP, cannot replace the acyl-CoA in the chemical reaction, but was able to acylate the Myr-Yes-C₃ peptide when incubated with ATP and CoA in the

Figure 3 Dependency of S-acylation on the concentrations of both substrates in the S-acylation reaction

(A) was obtained using different [³H]Pal-CoA concentrations. (B) was obtained by varying the Myr-Yes-C₃ concentration. This is a representative experiment repeated once with similar results. Insert in (B) is the fitting of the data to the Eadie-Hofstee equation to obtain a pseudo K_m and V_{max} for Myr-Yes-C₃; a subset of data points is shown for clarity.

presence of the P-100 fraction (data not shown). This result suggests that the actual substrate is probably acyl-CoA produced by acyl-CoA synthetase present in the membrane preparation.

Other factors that could affect any possible enzymatic reaction were also investigated. For example, increasing amounts of NaCl (0–300 mM) did not affect either the chemical acylation or the acylation in the presence of microsomes. Ba²⁺, Ca²⁺, Cd²⁺, Mg²⁺, Mn²⁺, Fe²⁺, Ni²⁺, Cu²⁺ and Zn²⁺ between 1 and 10 mM had no significant effect, either on the chemical acylation or when adding the microsomal fraction to the incubation mixture, except that at higher concentrations of Cu²⁺, Zn²⁺ and Fe²⁺ the production of labelled peptide dropped sharply, suggesting a non-specific inhibition perhaps due to metal binding to one of the substrates (data not shown). Triton X-100 at 0.1% caused an approx. 1.8-fold increase in non-enzymatic S-acylation (Table 2D). When Triton X-100 was added in the presence of COS P-100 fraction a reduction in the formation of product compared

Figure 4 Effect of ACBP on *in vitro* acylation of actin

Actin ($10 \mu\text{M}$) was incubated with increasing amounts of ACBP and $1.5 \mu\text{M}$ [^3H]Pal-CoA followed by resolution by SDS/PAGE. Coomassie staining is shown in the bottom panel and the corresponding fluorogram is in the top panel. The fluorogram shows that ACBP blocks the labelling of actin by [^3H]Pal-CoA even when present at only a slight molar excess over the acyl CoA. This is a representative experiment out of two replicates.

with that with Triton alone was observed. This may have been due to potentiation of membrane-associated thioesterase activity by detergent extraction (Table 2D).

Instead of the expected increase in reaction in the presence of a source of putative enzyme (microsomes), a decrease in the amount of labelled peptide was always observed. In order to explain this effect, two types of experiments were carried out. First, it was possible that when using membranes, part of the Pal-CoA or peptide was partitioning into the membranes, resulting in a sequestration and/or dilution of substrates. To test this we performed the acylation reaction with different amounts of SUV liposomes (DOPC:DOPE:DPPC, 2:1:1). The amount of labelled product decreased when increasing the liposome concentration (Table 2E). A similar but less dramatic effect was observed when microsomes were added to the samples. Alternatively, the decrease could be attributed to thioesterase activity. Myr-Yes-C3 peptide was incubated with [^3H]Pal-CoA in acylation buffer for 1 h to generate S-acylated product and then S-100 or P-100 fraction was added to the reaction for another hour at 37°C . An approx. 20% decrease was detected in the palmitoylated peptide when using $7.6 \mu\text{g}$ P-100 fraction and 10% when incubating with the same amount of S-100 fraction (data not shown). We conclude that both effects, substrate/product partitioning into the membranes and thioesterase ac-

tivity, may contribute to the observed decrease in the amount of labelled peptide on addition of membranes.

As shown by Knudsen and co-workers [44–46], the cytoplasmic ACBP binds acyl-CoA with chain lengths of 14–22 carbon atoms with high affinity and a possible role in regulating acyl-CoA distribution and fate has been suggested. To investigate whether a significant S-acylation of peptides can be detected *in vitro* in the presence of this protein, different amounts of ACBP were added to the acylation reaction. Table 2F shows that increasing ACBP concentration causes an initial decrease in the production of acylated peptide but that inhibition greater than 60% was never achieved. The molar excess of ACBP over [^3H]Pal-CoA varied between 100- and 1000-fold in these experiments. In control experiments using gel filtration, we confirmed that ACBP binds [^3H]Pal-CoA with high efficiency at a 1:1 molar ratio (data not shown). No significant difference was found in the presence of microsomes in the reaction.

Chemical acylation of proteins

In order to examine if the chemical reaction was also able to S-acylate reduced cysteines in proteins, actin (containing 5 cysteine residues) was used as a model. [^3H]Pal-CoA ($1.5 \mu\text{M}$) was incubated with actin ($10 \mu\text{M}$) in acylation buffer at 37°C for 60 min. Aliquots were loaded in duplicate on 12.5% polyacrylamide gels and stained with Coomassie Blue or treated for fluorography. A radioactive band corresponding to the actin was found (Figure 4, lane 0). This band was no longer detected after treating the gel with 1 M hydroxylamine overnight, prior to the DMSO incubation, suggesting that the ^3H label is attached to cysteine via a thioester bond (data not shown). A similar result was found when using a different cytosolic protein, lactate dehydrogenase, or a secretory protein, fetuin (data not shown). These data suggest a non-specific acylation reaction and *a priori* any protein containing reduced cysteine could react with fatty acyl-CoA and be modified. Therefore some system must operate *in vivo* to prevent these promiscuous acylation reactions. One candidate could be ACBP, and Figure 4 also shows that when ACBP is added to the acylation reaction (2–100 μM ; 1.33 to 66.7-fold molar excess) it abolishes the palmitoylation of the actin, presumably by binding and sequestering palmitoyl-CoA.

DISCUSSION

The cycle of S-acylation and deacylation observed for many proteins implies that activities responsible for each reaction exist [47]. Intuitively one might expect that both steps would be enzymatic and at least one would be regulated. However, in order to control such a simple cycle only one limb need theoretically be regulated and enzyme-catalysed. A thioesterase that is active on Ras and G-protein α -subunits has recently been purified [28] but subsequent cloning of the cDNA showed it to be a secreted protein and therefore not the authentic deacylating enzyme for cytoplasmic proteins [29].

The S-acylating activity has proved extremely refractory to purification despite long-term concerted efforts by several groups. This is consistent with earlier reports of non-enzymatic S-acylation of proteins and peptides *in vitro* [30–34,36] and with the results presented in this study. Efficient S-acylating activity can be observed without any protein-mediated catalytic or autocatalytic activity. Using synthetic peptides based on the N-terminal dual acylation motif of the c-Yes protein, we have observed an *in vitro* chemical acylation that has characteristics (time-, pH-, temperature-, and substrate concentration-dependence) that may allow it to operate *in vivo*. This can be highly

efficient, proceeding almost to completion under appropriate conditions. The reaction exhibits pseudo-enzymatic properties, and apparent K_m and V_{max} values can be estimated for the peptide substrate. Although all of the data in this paper were obtained *in vitro*, they are consistent with much of the data obtained *in vivo*. The acylation requires activated fatty acid (no reaction was found when using palmitic acid) but there is a relatively broad specificity for long chain (myristoyl or palmitoyl) acyl-CoA although acetyl-CoA was not a substrate. This result is in good agreement with the relatively broad specificity found in *in vivo* studies. Labelling of human platelets with either [3 H]palmitate or [3 H]arachidonate resulted in a hydroxylamine-sensitive incorporation of both labels into the α -subunits of G_i , G_q and G_z [13]. Such observations indicate that the G-protein pool may be heterogeneously acylated and may reflect differences in the acyl-CoA pool of individual cell types. The fatty acids which are transferred to Cys residues reflect to some extent the cellular abundance of acyl-CoA; palmitate ($C_{16:0}$) usually being predominant but stearate ($C_{18:0}$), oleate ($C_{18:1}$), retinoate and others also being found [5]. A recent report [6] has identified differences in the S-acyl groups attached *in vivo* to different viral surface glycoproteins. This could be interpreted as being evidence for acyl-CoA specificity of different protein:S-acyl transferase enzymes. An alternative explanation consistent with our data is that the substantially different cytoplasmic tails of these proteins 'select' different acyl-CoAs by a purely biophysical mechanism.

It is important to note that, in our experiments, peptides not containing N-terminal myristoylated glycine failed to be S-acylated, again mimicking the well-described dependence of S-acylation on prior membrane association via myristoyl, prenyl or transmembrane peptide moieties [5]. This suggests that some orientation or aggregation is required *in vitro*. Apparently micelle formation is not necessary for the S-acylation reaction to occur (see Results) but the myristoyl chain may facilitate an interaction with palmitoyl-CoA via acyl-acyl interactions in a determined orientation or mixed aggregate formation with acyl-CoA. The presence of 0.1% Triton X-100 in the reaction increases the chemical acylation but has no effect when P-100 fraction is used, possibly because the detergent helps to solubilize the substrates, thus favouring the interaction between them, or effectively solubilizes the very hydrophobic product of the reaction which is insoluble in aqueous buffer (M. C. Bañó, unpublished work).

In vivo, it has been found that there is no clear primary sequence requirement for S-acylation in the protein acceptor. The acylated Cys residues usually occur in regions close to the cytoplasmic surface of cellular membranes [48], being located there either by virtue of an associated transmembrane peptide sequence or another modification such as myristoylation or prenylation. From these studies it has been concluded that there is no apparent consensus sequence for the S-acylation site and no convincing consensus recognition sequences for any possible S-acyl transferase enzyme(s) have yet been discerned [47].

As described by Schroeder et al. [37], the acylation reaction is highly specific for cysteinyl over seryl or threonyl residues and for lipid-modified peptides over hydrophilic peptides. The authors suggest that the S-acylation process appears, by various criteria, including inhibition by tunicamycin, to be enzymatic. We also tried to inhibit the S-acylation reaction *in vitro* using this antibiotic, but no effect was observed (results not shown). Tunicamycin has been shown to inhibit protein S-acylation in cultured neuronal cells at relatively high concentrations [49] but it is not clear if this molecule directly inhibits an S-acyltransferase or another enzyme which is a prerequisite for the reaction to occur. Since the data of Schroeder et al. [37] were obtained *in vivo* and long chain acyl-CoA is the donor for S-acylation reactions,

their results do not exclude the possibility that the apparently enzymatic nature of the observed S-acylation is due to acyl-CoA synthetase activity rather than protein:S-acyl transferase activity.

After the characterization of a facile chemical S-acylation reaction, the possibility of the existence of an enzyme was tested. Adding freshly prepared subcellular fractions to the acylation reaction never resulted in an increase in the rate or extent of S-acylation. Even under conditions suboptimal for the chemical reaction but approximating to physiological conditions the extent of peptide acylation with cell fractions was always less than the purely chemical reaction. The decrease in the product could be explained by the activity of non-specific thioesterases (present in the membrane and cytosolic fraction) and because of the apparent sequestration and dilution of the substrate into the membranes, concordant with the results obtained with liposomes.

Chemical S-acylation has also been demonstrated for proteins. Actin (and other proteins) was S-acylated by a thioester bond when incubated with [3 H]palmitoyl-CoA, suggesting the non-specificity of the reaction. However, it is clear that this non-specific process does not operate *in vivo* and must be suppressed in some way. Presumably the amount of S-acylated protein formed depends on the number of sulphhydryls available for acylation and the amount of free acyl-CoA present at a particular location. Rasmussen et al. [44,45] have recently suggested that the dissociation constants for binding of long-chain acyl-CoA to cytosolic ACBP, and hence the concentration of free cytoplasmic long-chain acyl-CoAs, may lie in the femtomolar range under physiological conditions. These data are in good agreement with our results; S-acylation of peptide decreased somewhat with increasing ACBP concentration and S-acylation of actin was essentially abolished in the presence of approximately equimolar ACBP. The reason for the failure of ACBP to suppress peptide S-acylation completely even at a 1000-fold molar excess could be due to partial penetration of the ACBP-Pal-CoA complex by the small peptide whereas actin and other proteins would be excluded. All these data permit the conclusion that cytosolic proteins cannot be easily S-acylated *in vivo* because the free cytosolic pool of acyl-CoA molecules is very small; therefore, the only acyl-CoA available will be the membrane-associated pool. Approximately 10% of cellular palmitoyl-CoA is bound to membranes in COS cells (A. I. Magee, unpublished work). This is presumably associated with its acyl chain inserted in the lipid bilayer and its reactive head group oriented towards the cytoplasm. Only proteins that reach the membrane and have a Cys in an appropriate orientation and distance will be able to be palmitoylated. This could explain the prerequisite of myristoylation or prior protein localization in membranes for S-acylation to occur *in vivo*, in contrast with the hypothesis that rationalizes this association with the membranes as being necessary for the proteins to access a membrane-bound S-acyl-transferase.

All these results are consistent with the model depicted in Figure 5. *In vitro* the myristoyl group bound to the peptide could mediate orientation with the acyl chain of the CoA ester thus facilitating chemical reaction in a pseudo-enzymatic way. *In vivo*, proteins able to interact with membranes will react with the available pool of acyl-CoA, in an oriented manner. The distribution of long chain acyl-CoAs in cellular membranes is not well understood, but could play a role in the preferential S-acylation of proteins at certain sites such as the plasma membrane. Data in support of an enzymatic mechanism of S-acylation include the localization of this event to a specific subcellular compartment during biosynthesis of transmembrane proteins [37,50]. This could be explained by the necessity for the polypeptide acceptor to attain a suitable conformation which presents acceptor cysteine(s) to acyl-CoA, or by differential partitioning

in vitro *in vivo*

Figure 5 Hypothetical models for the S-acylation reaction

In vitro, myristoylated peptides may be able to orientate their acyl chain in concordance with the acyl chain of acyl-CoA, bringing the Cys sulphhydryl group close enough to react. *In vivo*, a similar situation could occur but the protein and acyl-CoA acyl chains would be inserted in the membrane which would act as a two-dimensional catalyst by concentrating the reactants.

of long chain acyl-CoAs into subcellular compartment membranes. In both cases, the lipid-modified protein would be immobilized in the membrane presenting the reactive cysteine to the acyl-CoA inserted in the membrane (Figure 5).

Recent papers [25–27] have provided further evidence for enzymatic protein:S-acyl transferase activity which is labile to heat and short term storage; however, no enzyme has been purified to homogeneity. Liu et al. [51] reported the purification of a S-acyl transferase activity acting on farnesylated Ras protein. A protein entity responsible for this activity has yet to be identified, and the ability of this activity to act on a variety of known S-acyl proteins is unclear. Similarly, Das et al. [52] have purified a 70 kDa protein which enhances S-acylation of spectrin but the mechanism of this effect is as yet unclear. The resolution of these questions will await the purification, sequencing and cloning of a protein capable of demonstrating *bona fide* S-acyl transferase activity when expressed as a recombinant molecule. A final proof will be the knockout of this gene in one or more organisms and the subsequent demonstration of loss of protein S-acylation. It is impossible to demonstrate the non-existence of an enzyme; until the existence of such an enzyme is proven the possibility that non-enzymatic S-acylation is a significant *in vivo* phenomenon must be considered viable. We have not attempted rigorously to mimic intracellular conditions in our experiments since this would entail making too many assumptions about the local environment of the S-acylation reaction and the con-

centration of substrates. However, a simple and thermodynamically possible chemical S-acylation can clearly occur in the absence of cellular fractions and this raises the question of whether an enzyme is required for the *in vivo* S-acylation of many proteins. Therefore, in the S-acylation cycle of proteins the regulated step may be the deacylation [53], which is essential to explain the biological reversibility of the process.

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