Palmitoylation of multiple Src-family kinases at a homologous N-terminal motif

Manfred KOEGL,* Philippe ZLATKINE,† Steven C. LEY,‡ Sara A. COURTNEIDGE* and Anthony I. MAGEE†§

*Differentiation Programme, EMBL, Postfach 10.2209, Heidelberg 69012, Germany, †Laboratory of Eukaryotic Molecular Genetics, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K., and ‡Laboratory of Cellular Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, U.K.

We have recently identified a novel N-terminal cysteine-containing motif which specifies the palmitoylation of several G-protein α -subunits [Parenti, Viganó, Newman, Milligan and Magee (1993) *Biochem. J.* **291**, 349–353]. A related motif occurs at the N-terminus of members of the Src family of protein tyrosine kinases except for Src itself and Blk. We have investigated whether the Src, Fyn, Yes and Lck gene products are palmitoylated. Src was not labelled with [3 H]palmitate when endogenously expressed in COS cells. In contrast, endogenous Yes immunoprecipitated from COS cells was palmitoylated. Fyn was palmitoylated in insect cells infected with a recombinant baculovirus and the palmitoylation was independent of protein synthesis, suggesting a dynamic turnover of this lipid. Fatty acid analysis indicated that most of the label was incorporated as palmitate. Lck was palmitoylated when expressed by transfection in COS cells. All of these protein tyrosine kinases were also

detectably myristoylated in each of the systems tested. Experiments performed with mutants of Lck expressed by transfection in COS cells indicated that cysteines at positions 3 and 5 were both palmitoylation sites and that myristoylation was required for palmitoylation. To confirm that palmitoylation was occurring on cysteines in the N-terminal region of Fyn, site-directed mutagenesis was used to replace the cysteines at positions 3 and 6 with alanine. The resulting protein was not palmitoylated but was still myristoylated when expressed in COS cells. A glycine to alanine mutant at position 2 was also not palmitoylated, showing that myristoylation is a prerequisite for palmitoylation. Our data indicate that Src family members containing the N-terminal cysteine motif are indeed palmitoylated. By analogy with Ras, it is possible that palmitoylation may play an important role in the localization and function of Src family protein tyrosine kinases.

INTRODUCTION

Protein acylations with fatty acids are important post-translational modifications that direct protein–membrane and protein–protein interactions [see Schlesinger (1993)]. We and others have recently identified a novel N-terminal motif in many G-protein α -subunits consisting of one or more cysteine residues within 18 residues of the initiator methionine, which can become post-translationally palmitoylated (Parenti et al., 1993; Linder et al., 1993; Degtyarev et al., 1993; Wedegaertner et al., 1993; Veit et al., 1994). In many instances, the N-terminus is also myristoylated on a glycine residue immediately after the initiator methionine. The most common motif of this kind comprises the sequence, initiator-Met-Gly-Cys. Searches of protein sequence databases using this sequence, and variants of it, identify approximately 250 proteins that are potential substrates for the same set of modifications (A. I. Magee, unpublished work). Of these, the most striking group of sequences corresponds to members of the Src family of protein tyrosine kinases (PTKs). This group of nine PTKs is defined by their similarity in sequence, topology and function [reviewed in Bolen (1993) and Courtneidge et al. (1994)]. The first 12 amino acids comprise a region important for fatty acylation and membrane localization (Resh, 1993). This is followed by the region that is most divergent between Src family members, called the unique domain, two domains directing protein–protein interaction (the SH2 and SH3 domains), the catalytic domain and a short tail important in negative regulation of kinase activity [reviewed in Cooper and Howell (1993)]. Several Src family kinases appear to be involved in signal-

transduction processes relaying signals from transmembrane or glycosylphosphatidylinositol (GPI)-anchored cell-surface receptor-like proteins to the cell interior [reviewed in Cantley et al. (1991) and Rudd et al. (1993)]. Furthermore, expression of constitutively activated alleles of Src family PTKs results in cell transformation, supporting the hypothesis that these proteins are involved in growth regulation (Cooper, 1990; Courtneidge et al., 1994). The attachment of a myristic acid moiety to Gly-2 is crucial for localization to the inner leaflet of the plasma membrane and for cell transformation by activated versions of Src family PTKs (Resh, 1993).

As all members of the Src family of PTKs, except for Src itself and Blk, showed homology to the initiator-Met-Gly-Cys motif (Figure 1), we set out to test whether these proteins were palmitoylated. As predicted, our data demonstrate that Fyn, Yes and Lck PTKs are palmitoylated whereas Src is not. We also confirm, using site-directed mutagenesis, that the N-terminal cysteines of Lck and Fyn are the acceptor residues for palmitate.

MATERIALS AND METHODS

Mammalian cell culture and metabolic labelling

COS 7 cells were maintained in Dulbecco's modified Eagle's medium plus 10% (v/v) fetal calf serum. For metabolic labelling of proteins, cells were transferred to the appropriate medium containing one-tenth of the normal concentrations of methionine and cysteine and labelled for 6–18 h with 20–100 μ Ci/ml Tran 35 S-label (ICN Biomedicals). For fatty acid labelling, the appropriate medium was prepared with dialysed serum and 5 mM sodium

Abbreviations used: PTK, protein tyrosine kinase; GPI, glycosylphosphatidylinositol.

§ To whom correspondence should be addressed.

Src	Met	Gly	Ser	Ser	Lys	Ser	Lys	Pro	Lys	Asp	Pro
Lck	Met	Gly	Cys	Val	Cys	Ser	Ser	Asn	Pro	Glu	Asp
Fyn	Met	Gly	Cys	Val	Gln	Cys	Lys	Asp	Lys	Glu	Ala
Yes	Met	Gly	Cys	Ile	Lys	Ser	Lys	Glu	Asp	Lys	Gly
Lyn	Met	Gly	Cys	Ile	Lys	Ser	Lys	Gly	Lys	Asp	Ser
Hck	Met	Gly	Cys	Met	Lys	Ser	Lys	Phe	Leu	Gln	Val
Yrk	Met	Gly	Cys	Val	His	Cys	Lys	Lys	Lys	Ile	Ser
Fgr	Met	Gly	Cys	Val	Phe	Cys	Lys	Lys	Leu	Glu	Phe
Blk	Met	Gly	Leu	Leu	Ser	Ser	Lys	Arg	Gln	Val	Ser

Figure 1 N-terminal sequences of Src-family members

The N-terminal 11 residues of members of the SRC family of PTKs are shown. Cysteine residues which are potential palmitoylation sites are emboldened. In the mature form of each protein the initiator methionine is removed and replaced by myristate.

pyruvate (FA medium). Cells were labelled in FA medium for 6–18 h with 100–300 $\mu\text{Ci/ml}$ [9,10- ^3H]palmitic acid (Amersham International, Amersham, Bucks., U.K.) or [9,10- ^3H]myristic acid (Du Pont–New England Nuclear). After labelling, cells were harvested in RIPA buffer (1% Nonidet P40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 150 mM NaCl, 50 mM Tris/HCl, pH 7.5) and immunoprecipitated as described previously (Magee et al., 1987). For some immunoprecipitations the primary antibody was covalently cross-linked to the Sepharose beads using dimethylpimelidate as described by Harlow and Lane (1988). Transfection of COS cells using electroporation has been described previously (Newman et al., 1993).

Antibodies

Antibodies specific for Fyn and Yes have been described previously (Kypta et al., 1988). Lck I antiserum was raised in rabbits against the C-terminal 20 amino acids of human Lck and demonstrated to be specific for Lck by Western-blot analysis of lysates of COS cells transfected with Lck or Fyn cDNAs in the pSM vector (R. Huby and S. C. Ley, unpublished work). Mouse monoclonal antibody 327 was used for Src immunoprecipitation (Lipsich et al., 1983).

Plasmids and site-directed mutagenesis

Mouse Lck cDNA and mutants LC1, LC2, LC1/2 and LC-G2, cloned into the pSM vector (Turner et al., 1990), were a gift from Dr. Dan Littman, (University of California at San Francisco). Fyn cDNA in the pSG5 vector (Green et al., 1988) was mutated by PCR-mediated site-directed mutagenesis. The sequence of the mutagenic primer is 5'-GATAATGGGCGCTGTGCAAGCT-AAGGAT-3'.

Baculovirus expression experiments

The construction of baculovirus vector directing the expression of Fyn has been described previously (Twamley et al., 1992). Passage and transfer of insect cells, selection and growth of plaques and virus infections were carried out as described in Summers and Smith (1987). For metabolic labelling with fatty acids, the cells were infected for 2 days, washed with PBS and resuspended in Graces medium (Summers and Smith, 1987). Cells were predepleted in Graces medium (for fatty acid labelling) or in methionine-free Graces medium (for ^{35}S -labelling) for 1 h and then incubated with ^3H -labelled fatty acids (500 $\mu\text{Ci/ml}$) or

Tran ^{35}S -label (10 $\mu\text{Ci/ml}$) for 2 h. Labelling was performed in 15 mm multidishes. Cells were extracted with lysis buffer {1% Nonidet P40, 20 mM Hepes, pH 7.2, 2 mM dithiothreitol, 10 mM NaF, 100 μM sodium orthovanadate [made up as described in Kypta et al. (1988)], 200 μM leupeptin, 1% aprotinin} and the extract was clarified by centrifugation at 13000 g for 5 min. Fyn was either immunoprecipitated with a specific rabbit antibody [Fyn2 (Kypta et al., 1988)] or the crude protein extract was directly loaded on an SDS/polyacrylamide gel (2×10^5 cell equivalents per lane). After electrophoresis, the gel was washed twice in water for 15 min, then immersed in 1 M salicylic acid for 1 h, dried and analysed by fluorography.

Miscellaneous methods

The method for hydrolysis of acylated proteins and analysis of fatty acids by t.l.c. has been described (Masterson and Magee, 1992). Methods for SDS/PAGE (Laemmli, 1970), hydroxylamine treatment and fluorography (Masterson and Magee, 1992; Magee et al., 1994) were also as published previously. The method for Triton X-114 partitioning is described in Gutierrez et al. (1989). All experiments were performed at least twice with similar results.

RESULTS

Palmitoylation of Yes, but not Src, in COS cells

To test for acylation of Src and Yes, COS cells, which express both kinases, were labelled with Tran ^{35}S -label, [^3H]palmitate or [^3H]myristate and immunoprecipitated with the appropriate specific antibodies (Figure 2). The Tran ^{35}S -labelled cells were dissolved in Triton X-114 and the detergent-enriched phase was used for immunoprecipitation to reduce the background. This phase contained the majority of the Src and Yes proteins (results not shown). [^3H]Myristate label was incorporated into both Yes and Src, whereas only Yes incorporated detectable [^3H]palmitate label. Duplicate lanes were treated with hydroxylamine to cleave thioester-linked fatty acids. This treatment removed the [^3H]palmitate label from the Yes protein, but had no effect on [^3H]myristate labelling of Src or Yes, indicating that interconversion of the fatty acids was not significant in the COS cells under these conditions.

Palmitoylation sites of Lck

The T-lymphocyte-specific kinase Lck has recently been reported to be palmitoylated in Jurkat J6 cells (Paige et al., 1993). In order to investigate the sites of palmitoylation of Lck, we took advantage of mutants that had been generated for the purpose of examining the role of cysteine residues in the unique region of Lck in interactions with CD4 (Turner et al., 1990). In mutants LC1 and LC2 the cysteines in positions 3 and 5 were replaced by serine and lysine respectively. Mutant LC1/2 contains both of these mutations, whereas mutant LC-G2 has a glycine to alanine mutation at residue 2 to remove the myristoylation signal. These mutants, as well as the wild-type protein, were expressed by transfection in COS cells (Figure 3). All the proteins were expressed at comparable levels. The LC-G2 mutant migrated more slowly than the wild-type protein. The LC2 and LC1/2 mutants also showed a significant amount of a more slowly migrating band. Wild-type, LC1, LC2 and LC1/2 proteins were all myristoylated, although the labelling of LC2 and LC1/2 was less than that of the wild-type. As expected, no [^3H]myristate labelling of the LC-G2 mutant was detected; in addition, no labelling was observed of the upper band of the LC2 and LC1/2

Figure 2 Acylation of PTKs in COS cells

COS cells were labelled with [^3H]palmitate (Pal), [^3H]myristate (Myr) or Tran ^{35}S -label (Met). Tran ^{35}S -labelled cells were lysed in Triton X-114 and the detergent-enriched phase was prepared. Fatty acid-labelled cells were lysed in RIPA buffer. All samples were precleared with $3\ \mu\text{l}$ of non-immune rabbit serum plus $10\ \mu\text{l}$ of Protein A-Sepharose beads. All the supernatants were then immunoprecipitated with normal rabbit serum (n), Yes-3 antiserum (y) or Src monoclonal antibody 327 (s) and resolved by SDS/PAGE (10% gel), followed by fluorography with 2,5-diphenyloxazole/dimethyl sulphoxide. The fatty acid-labelled immunoprecipitations were performed in duplicate and one set of lanes was treated with 1 M hydroxylamine for 1 h at room temperature. The other set of lanes was treated with 1 M Tris/HCl as a control. The migration positions of Src and Yes are shown.

Figure 3 Palmitoylation sites of Lck

COS cells were transfected with cDNA constructs encoding wild-type Lck (wt) or mutants LC1, LC2, LC1/2 and LC-G2. A mock transfection (m) was also performed. At 48 h after transfection the cells were labelled with Tran ^{35}S -label (Met), [^3H]palmitate (Pal) or [^3H]myristate (Myr), lysed and immunoprecipitated with an antiserum against the Lck C-terminus. The immunoprecipitates were resolved by SDS/PAGE (10% gel) followed by fluorography with 2,5-diphenyloxazole/dimethyl sulphoxide for 25 days (Pal and Myr) or sodium salicylate for 14 days (Met). The dark blemish in the top right hand corner of the Pal panel is not a band on the gel, but is an artifact that appeared during exposure of the fluorogram.

doublents. Wild-type Lck was efficiently labelled with [^3H]palmitate, whereas the LC1 and LC2 mutants were labelled to a lesser extent. Neither the LC1/2 nor the LC-G2 proteins were labelled with palmitate. These data suggest that cysteines at positions 3 and 5 are both palmitoylation sites and can account for all the [^3H]palmitate incorporation into Lck. In addition, myristoylation appears to be required for palmitoylation to

Figure 4 Palmitoylation of Fyn in baculovirus-infected insect cells

(a) Palmitoylation and myristoylation of Fyn in insect cells. Sf9 cells were infected with recombinant baculovirus expressing Fyn labelled with ^3H -labelled fatty acids or [^{35}S]methionine as indicated and immunoprecipitated with Fyn2 antiserum (lanes marked +). In the lanes marked -, the antibody had been preblocked with the antigenic peptide. The precipitated proteins were separated by PAGE followed by fluorography with 2,5-diphenyloxazole/dimethyl sulphoxide. (b) Demonstration that palmitoylation is not dependent on protein synthesis. Infected insect cells were labelled as in (a) after treatment with 100 mg/ml cycloheximide as indicated. Whole cell lysates were analysed as in (a).

occur. The appearance of a more slowly migrating unmyristoylated band, corresponding in mobility to the LC-G2 mutant, in the LC2 and LC1/2 mutants also suggests that mutations in the N-terminal region, particularly of Cys-5, may compromise the efficiency of the myristoylation reaction. Indeed, mutations of the N-terminal cysteines of Fyn to tyrosines interfered with myristoylation of Gly-2 (results not shown).

Palmitoylation of Fyn in baculovirus-infected insect cells

It has previously been shown that Src can be efficiently produced in Sf9 insect cells and that myristoylation of Src occurs in such cells (Pawson-Worms et al., 1990). We employed the baculovirus expression system to test whether Fyn became palmitoylated as well as myristoylated in insect cells. Sf9 cells were infected with a virus that contained Fyn cDNA under the control of the strong polyhedrin promoter. Metabolic labelling with ^3H -labelled myristic and palmitic acids showed that Fyn incorporated both fatty acids (Figure 4a). The identity of the labelled moiety was analysed by t.l.c. to assess the metabolic conversion of the radiolabelled fatty acids. Fyn immunoprecipitates and total protein from Sf9

Figure 5 Requirements for palmitoylation of Fyn

(a) Vectors expressing the indicated cDNAs were transfected into COS cells and incubated with ^3H -labelled myristate (M) or palmitate (P). After cell lysis in RIPA buffer, Fyn protein was isolated by immunoprecipitation with Fyn2 antiserum and analysed by SDS/PAGE followed by fluorography with 2,5-diphenyloxazole/dimethyl sulphoxide. (b) Western blot of the same transfections using anti-Fyn2 serum.

cells infected with the Fyn virus and labelled with ^3H palmitate or ^3H myristate were precipitated with acetone and washed extensively with acetone and chloroform/methanol/water (65:25:4, by vol.) to remove non-covalently associated lipids. The pellets were hydrolysed in 6 M HCl at 110 °C and the released lipids extracted into hexane, followed by analysis by reversed-phase t.l.c. and fluorography (Masterson and Magee, 1992). After being labelled with ^3H palmitate, approx. 10–30 % of the protein-bound label had been converted into myristate, the balance remaining as palmitate. Conversely, after ^3H myristate labelling, approx. 30 % of the incorporated radiolabel had been converted into palmitate. When the total lipid fraction of the cells was analysed, approx. 50 % of ^3H myristate had been converted into palmitate, whereas only about 10 % of ^3H palmitate had been converted into myristate. Thus, although inter-conversion of radiolabelled fatty acids had occurred in this expression system, the bulk of the protein-bound label remained the same as the input fatty acid.

Attachment of myristic acid to proteins including Src occurs co-translationally and is blocked by inhibition of protein synthesis (Buss et al., 1984; Magee and Courtneidge, 1985). However, it has been shown that palmitoylation of the small GTP-binding protein Ras is not linked to protein synthesis (Magee et al., 1987). To test whether palmitoylation of Fyn was dependent on protein synthesis, we performed metabolic labelling of the cells in the presence of concentrations of cycloheximide that blocked the *de novo* synthesis of Fyn by 95 % (results not shown). As can be seen in Figure 4(b), cycloheximide treatment reduced incorporation of myristic acid at least fivefold, whereas the incorporation of palmitic acid was not affected. These data suggest that the attachment of palmitic acid to Fyn is not co-translational and occurs by a different mechanism from the attachment of myristic acid.

Mutational analysis of N-terminal residues required for palmitoylation of Fyn

By analogy with palmitoylation of G_α subunits (Parenti et al., 1993) and Lck, we predicted that the two N-terminal cysteine residues of Fyn were likely candidates as sites of palmitoylation. The fact that Src, which lacks these cysteines in its N-terminal unique region, does not become palmitoylated supports this hypothesis. To test this, the coding region of the cDNA for human Fyn was mutated such that the codons for the two cysteines were changed to alanines. In addition, a mutant cDNA in which Gly-2 had been changed to alanine and which was therefore unable to be myristoylated was also included in the analysis. The cDNAs were subcloned into the pSG5 vector and transfected into COS cells. After metabolic labelling of the cells, the gene products of the mutated cDNAs were immunoprecipitated and analysed by SDS/PAGE and fluorography. Mutation of the two cysteines abolished palmitoylation, but had no effect on myristoylation of Fyn (Figure 5). However, the myristoylation-deficient protein became neither myristoylated nor palmitoylated. Thus these data indicate that palmitoylation of Fyn depended on prior myristoylation, whereas palmitoylation was not required for the attachment of myristic acid. The observed loss of labelling was not due to different expression levels of the three protein variants, as comparable amounts of Fyn were detected by Western blotting (Figure 4b).

DISCUSSION

The N-terminal cysteine-containing regions of many members of the Src family are homologous to the cysteine residues that are palmitoylated in the N-terminal regions of G-protein α -subunits. The data presented here confirm that the Yes, Fyn and Lck PTKs are palmitoylated, whereas Src itself, which does not contain a cysteine residue near the N-terminus, is not. While this study was in progress two papers were published that also demonstrate palmitoylation of Lck (Paige et al., 1993; Shenoy-Scaria et al., 1993). Shenoy-Scaria et al. (1993) further showed the requirement of a pair of cysteines at the N-terminus of Lck for this modification. The data presented here confirm the palmitoylation of Lck and in addition demonstrate that Fyn and Yes but not Src become palmitoylated. Furthermore, we show that each of the two N-terminal cysteines of Lck can be palmitoylated independently of the other. We also report that abolition of myristoylation in the LC-G2 mutant of Lck blocks palmitoylation. Replacement of both N-terminal cysteines of Fyn with alanine also abolishes palmitoylation, as does mutation of the myristoylation site. These data indicate a requirement of myristoylation for palmitoylation to occur. In the case of the GTP-binding regulatory protein G_α , a defect in myristoylation also blocks the attachment of palmitic acid (Hallak et al., 1994). This situation is reminiscent of Ras proteins, where palmitoylation of C-terminal cysteines is dependent on prior prenylation of the cysteine of the adjacent CXXX motif (Hancock et al., 1989, 1990).

The dependence of palmitoylation on myristoylation of Lck and Fyn may reflect the requirement for interaction with membranes containing palmitoyltransferase activity (Gutierrez and Magee, 1991). However, myristoylation is not universally required to achieve palmitoylation of N-terminal cysteine residues, as the α_s , α_q , α_{12} and α_{13} proteins are not myristoylated but still undergo palmitoylation (Degtyarev et al., 1993; Wedegaertner et al., 1993; Veit et al., 1994). It may be that other mechanisms, such as protein–protein interactions, mediate the initial membrane association of these proteins, which allows their presentation to a palmitoyltransferase.

In a number of cases palmitoylation of proteins is dynamic (Magee et al., 1987; Paige et al., 1993). This has led to the suggestion that palmitoylation may regulate protein localization and function. We show here that palmitoylation of Fyn in insect cells is resistant to inhibition of protein synthesis, suggesting that it is also dynamic. Palmitoylation is important for membrane localization of Ras proteins (Hancock et al., 1989, 1990). Turner et al. (1990) showed that alteration of the cysteines in positions 3 and 5 of Lck, shown here to be the palmitoylation sites, or of the myristoylation site essentially abolished membrane binding. Thus palmitoylation of Lck is also important for membrane binding. Reversible palmitoylation of Lck and Fyn may be regulated during T-cell activation and may modulate the association of these proteins directly with the lipid bilayer, or with other proteins with which they are known to interact (Rudd et al., 1993). Indeed, association of Fyn and Lck with the GPI-anchored protein decay-accelerating factor requires intact palmitoylation sites (Shenoy-Scaria et al., 1993). As the GPI anchor is restricted to the outer leaflet of the lipid bilayer and the palmitate moiety does not extend beyond the inner leaflet, this interaction probably involves an intermediate transmembrane protein. Such a protein may also play a role in localizing the palmitoylated proteins to particular subcompartments of the plasma membrane, such as the Triton X-100-insoluble membrane fraction. We have found that tyrosine phosphorylation of cell proteins by Fyn differs from that induced by palmitoylation-deficient Fyn (results not shown), which is consistent with the idea that targeting of the PTK is modified by acylation. It is interesting to note that Lck associates with membranes after expression by transfection in COS cells lacking CD4 or CD8 (Turner et al., 1990) suggesting that membrane association could be directly with the lipid bilayer. This is consistent with recent data showing that the N-terminal region of Src family members probably does not interact with a specific protein receptor (Resh, 1993). Rather it may be that myristate, palmitate and basic residues co-operate in an as yet ill-defined manner in different Src family members to mediate membrane insertion and ionic interaction with acidic phospholipids and that protein-protein interactions also contribute to specific localization. This scenario is again similar to that characterized for the Ras family (Hancock et al., 1990, 1991).

In summary, N-terminal palmitoylation is a widespread modification of Src-related proteins which probably applies to all family members containing N-terminal cysteines. Palmitoylation of the unique region may play a role in subcellular localization and regulation of Src family PTKs. This is currently being investigated.

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