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Retargeting of cytosolic proteins to the plasma membrane by the Lck protein tyrosine kinase dual acylation motif

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SUMMARY

Several members of the Src family of protein tyrosine kinases have a N-terminal dual acylation motif which specifies their myristoylation and S-acylation. These lipid modifications are necessary for correct intracellular localisation to the plasma membrane and to detergent-resistant glycolipid-enriched membrane domains (GEMs). Using chimaeras of the Lck dual acylation motif with two normally cytosolic proteins (chloramphenicol acetyl transferase and galectin-3), we show here that this motif is sufficient to encode correct lipid modification and to target these chimaeras to the plasma membrane, as demonstrated by subcellular fractionation and confocal immunofluorescence microscopy of transiently transfected COS cells. In addition, the chimaeras are resistant to extraction with cold non-ionic detergent, indicating targeting to GEM subdomains in the plasma membrane. The dual acylation motif has potential for targeting proteins to specific plasma membrane subdomains involved in signalling.

Key words: Dual acylation, Myristoylation, S-acylation, Lck

INTRODUCTION

Protein acylations with fatty acids are important post-translational modifications that direct protein-membrane and protein-protein interactions (Schlesinger, 1993). A novel N-terminal motif consisting of one or more cysteine residues within 18 residues of the initiator methionine was recently identified in many G protein α subunits and members of the Src family of protein tyrosine kinases (PTKs). The most common motif of this kind is composed of the sequence, initiator-Met-Gly-Cys-X_n-(Cys) in which the cysteines become post-translationally S-acylated (Parenti et al., 1993; Koegl et al., 1994; Resh, 1994). In many instances, the N terminus is also myristoylated on the glycine-2 immediately after the initiator methionine, contributing to a dual acylation motif. The Src family PTKs Yes, Fyn and Lck are both myristoylated and S-acylated whereas Src itself, which does not contain a cysteine residue near the N terminus, is only myristoylated (Koegl et al., 1994; Shenoy-Scaria et al., 1993). S-acylation is dependent on prior co-translational myristoylation of Lck and Fyn and is required for membrane attachment (Turner et al., 1990), reminiscent of the situation for Ras proteins (Hancock et al., 1989, 1990).

The targeting of Src family PTKs to specific membrane compartments by attached lipid groups may play an important role during T cell activation by facilitating lipid-protein or protein-protein interactions. Glycosyl phosphatidylinositol (GPI)-anchored membrane proteins and the PTKs Lck and Fyn independently associate with a cold Triton X-100-insoluble glycolipid-enriched membrane fraction in a variety of cell types (Rodgers et al., 1994; Shenoy-Scaria et al., 1994). This may explain the ability of GPI-anchored proteins to stimulate the kinase activity of Src family PTKs (Zhou et al., 1995; Field et al., 1995). The GPI anchor was found to be the targeting signal to the glycolipid enriched microdomains (GEMs) in GPI-anchored proteins (Rodgers et al., 1994) while the amino-terminal sequence Met-Gly-Cys is necessary and sufficient within the context of Src family PTKs to confer localisation to cold Triton X-100-resistant membrane complexes, and S-acylation is essential (Shenoy-Scaria et al., 1993, 1994).

As a common membrane targeting signal is present at the amino-termini of different protein families (Koegl et al., 1994; Parenti et al., 1993; Shenoy-Scaria et al., 1994; Rodgers et al., 1994), we set out to test whether the Lck N-terminal dual acylation motif itself is a major determinant of protein localisation to plasma membrane and to GEMs. Our data demonstrate that the 10 amino acid N-terminal sequence of Lck is sufficient to confer localisation to the plasma membrane when fused to cytosolic proteins. The chimaeras also enter a Triton X-100-insoluble fraction which is promoted by S-acylation. The dual acylation motif can therefore be used as a general targeting signal to take proteins to these plasma membrane domains specialised for signalling (Lisanti et al., 1994).

MATERIALS AND METHODS

Materials

Tran ^{35}S-label (Specific activity 1,057 Ci/mmol) was obtained from ICN Biomedicals, High Wycombe, UK; [9,10-^{3}H]palmitic acid (30-60 Ci/mmol) and [9,10-^{3}H]myristic acid (30-60 Ci/mmol) were from Amersham. Rabbit polyclonal antiserum was raised against pure hamster galectin-3 as described (Mehul et al., 1994). Rat monoclonal antibody

M3/38 (anti-Mac-2) against mouse galectin-3 was from Boehringer Mannheim, East Sussex, UK. Antibody to caveolin was obtained from Affiniti Research Products Ltd, Nottingham, UK. Wheat germ agglutinin (WGA)-fluorescein and anti-rat and anti-rabbit IgG-fluorescein or Texas Red conjugates produced in goat (affinity-isolated antibody) were from Sigma. Anti-chloramphenicol acetyltransferase (CAT) rabbit polyclonal antibody was from 5 Prime-3 Prime Inc., Colorado, USA. Polyclonal antisera to full length human Lck expressed as a glutathione S-transferase (GST) fusion protein using the pGEX system (Pharmacia; a kind gift from Dr S. Ratnovsky, BASF, USA) were raised in rabbits (S. Ley and A. I. Magee, data not shown). These were highly specific for Lck by western blotting and immunoprecipitation against LSTRA T cell lymphoma and Cos-7 cells transfected to express Lck or Fyn (Koegl et al., 1994). All other biochemicals used were of the highest purity available and obtained from regular commercial sources.

Immunofluorescence

Transfected cells were plated on glass coverslips and grown for 24-48 hours, then washed three times with phosphate buffered saline (PBS) and fixed with methanol at −20°C for 8 minutes. Cells were washed twice with PBS and incubated with PBS containing 3%(w/v) bovine serum albumin (BSA) for 20 minutes then with PBS containing 1% BSA and primary antibodies (polyclonal anti-CAT 1:50; polyclonal anti-Lck, 1:50; monoclonal anti-Mac-2 antibody, 1:50) for 2 hours. Cells were washed five times with PBS and once with PBS-1% BSA then incubated for 1 hour with a secondary affinity-purified fluorescein-conjugated goat anti-rabbit or anti-rat IgG antibody (1:50 in PBS-1% BSA). Cells were extensively washed with PBS and slides were mounted in hydromount (National Diagnostics, UK) then examined with a Bio-Rad Lasersharp MRC600 imaging system attached to an Olympus BH2 microscope using Zeiss ×40 or ×63 planapochromat lenses. Untransfected cells were observed as negative controls. For double staining, WGA-fluorescein (2 μg/ml) was added with the second antibody (IgG-Texas Red conjugate).

Plasmid construction and transfection

A CAT cDNA was generated by PCR using Biotaq polymerase (Bioline, London, UK), with the primers 5′ ATAGTCGACATGGAGAAAAAAATCACTGGA 3′ and 5′ ATAGGATCCTTACGCCCCGCCCTG 3′, and CAT vector (Promega) as template using the following conditions: one cycle for 3 minutes at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, annealing at 45°C for 1 minute, extension at 72°C for 1 minute and finally 1 cycle at 72°C for 5 minutes. The single PCR product of 600 bp was digested with *Sal*I-*Bam*HI restriction enzymes and agarose gel purified. Galectin-3 (CBP 30) cDNA was isolated from the Q5 clone as described previously (Mehul et al., 1994) by *Sal*I-*Bam*HI restriction. Isolated galectin-3 cDNA and CAT cDNA were subcloned into the pSG5 plasmid (Koegl et al., 1994) using the *Eco*RI-*Bam*HI restriction sites and N-terminal Lck or mutated N-terminal Lck adaptors. These adaptors were generated by annealing of two primers; 5′ AATTCATGGGCTGTGGCTGCAGCTCACACCCGGAAGATG 3′ and 5′ TCGACATCTTCCGGGTGTGAGCTGCAGCCACAGCCCATG 3′ (for wild-type Lck N terminus) or 5′ AATTCATGGGCGCAGGCGCAAGCTCACACCCGGAAGATG 3′ and 5′ TCGACATCTTCCGGGTGTGAGCTTGCGCCTGCGCCCATG 3′ (for the C3A, C5A mutated Lck N terminus where underlining corresponds to the mutant alanine codons). All plasmids were used to transform *E. coli* DH5α and a large scale preparation of DNA was obtained and purified using a Qiagen kit. Nucleotide sequence was verified by the dideoxy-chain termination method using 5′ GCTCCTGGGCAACGTGCTG 3′ as a primer and [alpha-^{35}S]dATP (1,000 Ci/mmol, Amersham Corp.) using a Sequenase Kit, version 2.0 (U.S. Biochemical Corp.). Transfection was performed in Cos-7 cells by electroporation using 10 μg plasmid DNA (Newman et al., 1992).

Metabolic labeling, harvesting and immunoprecipitation

Transfected cells were grown for 24 to 48 hours at 37°C with 5% CO_2 in monolayer culture in Eagle's medium supplemented with 10%(v/v) foetal calf serum (FCS), 100 i.u./ml penicillin and streptomycin. For labeling, the cells were incubated for 16 hours at 37°C with methionine and cysteine-free Eagle's medium containing 10% dialyzed FCS, antibiotics and 25 μCi/ml of Trans ^{35}S-label. For fatty acid labelling, cells were incubated for 16 hours in Eagle's medium supplemented with dialyzed FCS, antibiotics and [9,10^{3}H]palmitate (200 μCi/ml) or [9,10^{3}H]myristate (200 μCi/ml) from a stock at 10 μCi/μl in ethanol.

For cell fractionation, cells were washed twice after labeling with PBS, harvested and spun down. The supernatants were discarded and the pellet was homogenized in a micro-homogenizer system (Biomedix, UK) in 300 μl of 10 mM Tris-HCl, pH 7.6, containing 1 mM EDTA, 1 mM PMSF and 100 mM thiodigalactoside (used to prevent binding of galectin-3 to glycoconjugates; Sato and Hughes, 1994). After low speed centrifugation to remove nuclei and intact cells, supernatants were centrifuged at 250,000 ***g*** for 30 minutes at 4°C. Supernatants were kept on ice and pellets were washed once with 300 μl of the same buffer and centrifuged again. The two supernatants were pooled (S) and added to 400 μl of RIPA buffer (50 mM Tris-HCl, pH8, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% Na deoxycholate, 0.1% SDS, 1 mM PMSF). The pellets (P) were resuspended in 1 ml of RIPA buffer.

Alternatively, cells were directly lysed in RIPA buffer (1 ml) after washing with PBS, incubated on ice for 30 minutes and then cleared by centrifugation at 12,000 ***g*** for 10 minutes at 4°C. Each fraction was precleared with 40 μl of a 50% (w/w) suspension of Protein A-Sepharose in RIPA buffer containing 5 μl of normal rabbit serum for at least 2 hours at 4°C then centrifuged for 6 minutes at 6,500 ***g*** at 4°C. The cleared supernatants were incubated overnight at 4°C with appropriate polyclonal antibodies (1:100) in the presence of 40 μl of 1:1 Protein A-Sepharose suspension. For Lck immunoprecipitation, polyclonal anti-Lck serum was precoupled with dimethyl pimelidate to Protein A-Sepharose as previously described (Harlow and Lane, 1988) due to interference of the antibody heavy chain with the migration of the Lck band.

The beads with bound immunocomplexes were washed five times with RIPA buffer (1 ml) for 10 minutes at 4°C then once with 50 mM Tris-HCl, pH 6.8, and heated in Laemmli sample buffer (Laemmli, 1970). Samples were separated by SDS-polyacrylamide gel electrophoresis (10%, 12.5% or 15%). Fluorography of dried 2,5-diphenyloxazole-treated gels was performed using preflashed Kodak X-Omat XAR5 film (Magee et al., 1995). Fluorography times were as follows: ^{35}S-labelled samples, 3-15 days; ^{3}H-labelled samples, 7 days-9 weeks.

Preparation of cold Triton X-100-insoluble plasma membrane domains (GEMs)

Labeled cells were washed twice with ice-cold PBS and centrifuged at 4°C. Cells were homogenized in the presence of thiodigalactoside (100 mM) in 300 μl of 50 mM Tris-HCl, pH 8, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1 mM PMSF (TTNE buffer) for 20 minutes on ice then centrifuged at 250,000 ***g*** for 30 minutes at 4°C. Supernatants (S1; cold Triton X-100-soluble fraction) were added to RIPA buffer (600 μl) and kept on ice. Pellets were washed twice with the same buffer (400 μl) at 4°C then resuspended in 300 μl TTNE buffer containing thiodigalactoside (100 mM), leupeptin and aprotinin (100 μM each) and incubated for 15 minutes at 37°C. Samples were centrifuged at 250,000 ***g*** for 30 minutes at 30°C. Supernatants (S2), which correspond to the 37°C Triton X-100-soluble fraction, were diluted in 600 μl RIPA buffer.

RESULTS

Membrane association of Lck, CAT and Galectin-3 fusion proteins

Fig. 1 shows the constructs used in these experiments. We set out to ask whether the Lck dual acylation motif is sufficient to

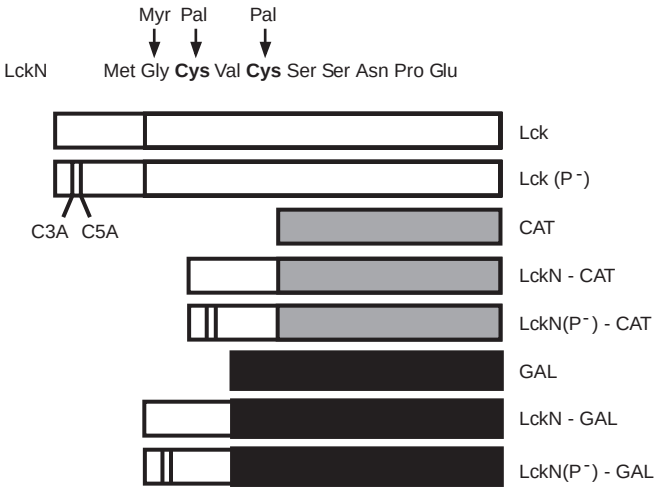

Fig. 1. Constructs of Lck and the Lck N terminus (LckN) with CAT and galectin-3 (GAL). P^- signifies mutation of cysteines 3 and 5 to alanine.

specify targeting to plasma membrane and to GEMs. As shown in Fig. 2, in which soluble (S) and membrane (P) fractions are compared, transfected wild-type Lck is palmitoylated (B) and binds predominantly to membranes (A) whereas replacement of Cys 3 and 5 by alanine (Lck(P^-)) causes the protein to be non-palmitoylated (Koegl et al., 1994) and to remain mostly soluble (A). Mutation of glycine-2, which was shown to prevent both myristoylation and S-acylation (Koegl et al., 1994), completely abolished Lck binding to the pellet fraction (data not shown). In order to determine whether the Lck N terminus is sufficient to confer the ability to bind proteins to cell membranes, constructs were made that encode two different reporter proteins fused to the 10 N-terminal amino acids of Lck (LckN-CAT and LckN-Gal). Chloramphenicol acetyl transferase (CAT) was chosen as a reporter protein because it is cytosolic when expressed in eukaryotic cells and is very stable. We chose galectin-3 to construct the second chimaera because it is a cytosolic protein although, unlike CAT, it is constitutively secreted at a slow rate from some cells, e.g. macrophages, by a novel pathway (Sato and Hughes, 1994) and therefore has some ability to cross the plasma membrane without being stably associated with it. When expressed in Cos-7 cells (Fig. 2A) both wild-type CAT and galectin-3 were located in the soluble fraction and were neither myristoylated nor S-acylated (data not shown). By contrast the chimaeric proteins LckN-CAT and LckN-Gal containing the wild-type Lck N terminus were S-acylated (Fig. 2B) and mainly membrane associated (Fig. 2A). The constructs made with either CAT or galectin-3 and the Lck N terminus mutated at cysteine residues 3 and 5 (LckN(P^-)-CAT and LckN(P^-)-Gal) lost their ability to be S-acylated (data not shown) and showed the opposite distribution pattern between the soluble and the pellet fraction when compared to the proteins bearing the wild-type motif, being found for the most part in the soluble fraction (Fig. 2A). Experiments using tritiated fatty acids showed that nearly all the S-acylated proteins were bound to the membranes (Fig. 2B), whereas a much reduced amount of the non-S-acylated but myristoylated proteins remained associated with the membranes (Fig. 2C).

Immunofluorescence staining of Lck, CAT and Galectin-3 fusion proteins expressed in Cos-7 cells

The subcellular localisation of wild-type Lck, galectin-3 and CAT and of the chimaeras and mutants expressed in transfected Cos-7 cells was also examined by confocal immunofluorescence microscopy. Wild-type Lck localised to the plasma membrane (Fig. 3A and C). In the population of transfected cells this plasma membrane staining was most commonly continuous as shown but sometimes had a more patchy character indicative of a non-uniform distribution in the plane of the membrane. A vesicular stucture adjacent to the nucleus was also brightly stained. Double staining experiments using WGA as a marker of the Golgi complex showed a partial colocalisation with Lck (Fig. 3B and C). As expected galectin-3 and CAT showed a diffuse pattern of staining characteristic of cytosolic proteins (Fig. 3E and J); there was no detectable staining in plasma membrane or intracellular vesicular structures. By contrast LckN-CAT and LckN-Gal which were myristoylated and S-acylated on the N-terminal motif showed a distribution very similar to wild-type Lck with distinct labelling in the plasma membrane (Fig. 3F and K) and accumulation in the perinuclear vesicular structure as shown by double staining using WGA (Fig. 3G,H,L and M). Fusion of the Lck dual acylation motif to both proteins drastically reduced the cytosolic staining. In contrast the LckN(P^-)-CAT and LckN(P^-)-Gal mutants, which were no longer S-acylated, showed no staining in the plasma membrane (Fig. 3I and N) and resembled the C3,5A mutated Lck(P^-) (Fig. 3D) and the wild-type galectin-3 and CAT (Fig. 3E,J) in their distribution. From these results it was clear that addition of a dual acylation motif to both CAT and galectin-3 promoted strong plasma membrane association. Mutation of the cysteine residues in positions 3 and 5 shifted the plasma membrane localisation of the Lck PTK and the chimaeric constructs to a cytosolic distribution.

S-acylation determines the ability of chimaeric proteins to be targeted to cold Triton X-100-insoluble plasma membrane domains (GEMs)

Lck has been found to be associated with cold non-ionic detergent-resistant membrane complexes (GEMs) in a variety of cells (Bohuslav et al., 1993; Rodgers et al., 1994; Shenoy-Scaria et al., 1994). These complexes are enriched in GPI-anchored proteins, cholesterol and glycolipids. In order to investigate whether the acylated N-terminal motif is the major determinant for Lck targeting and association with these domains, the distribution of wild-type proteins and chimaeras between the soluble fraction and high speed pellet was determined after cell extraction with buffer containing 1% Triton X-100 on ice. [^{35}S]Methionine labeling (Fig. 4A) showed that over half of the wild-type Lck was found in the GEM fraction S2 as previously reported in other cell types (Rodgers et al., 1994) and a similar proportion of the [^{3}H]palmitate-labelled Lck was able to associate with GEMs (Fig. 4B). By contrast Lck(P^-) (Fig. 4A) or Lck with glycine 2 mutated to alanine (data not shown), which are deficient, respectively, in S-acylation or myristoylation and subsequent S-acylation (Koegl et al., 1994), do not associate with GEMs. The same experiments repeated with either wild-type CAT or galectin-3 proteins or with the chimaeric constructs showed that fusion of the acylated Lck N-terminal motif to both cytosolic proteins leads to the partition-

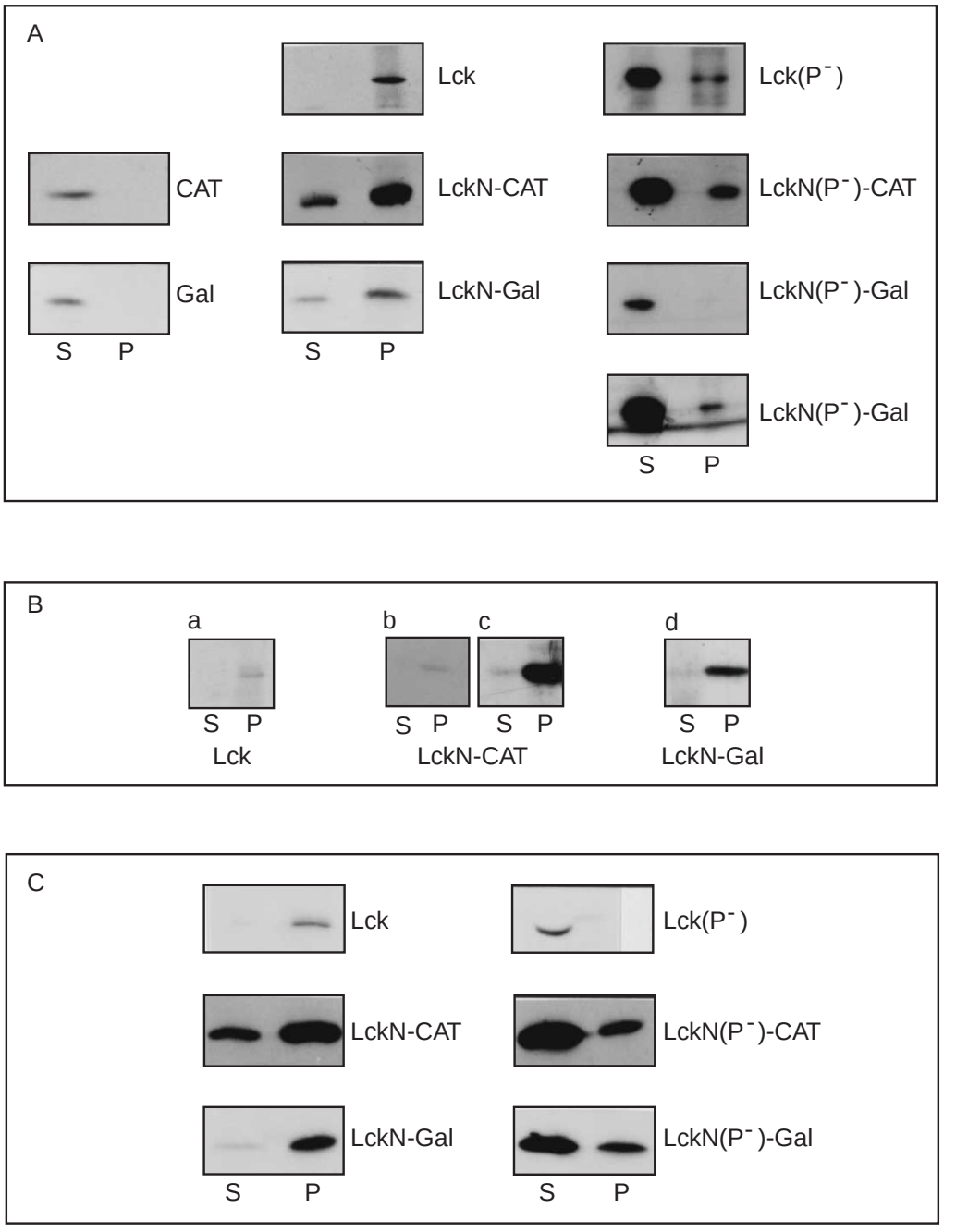

Fig. 2. Subcellular distribution of Lck and chimaeric constructs. (A) Transfected Cos cells were labelled with Tran ^{35}S-label and separated as described in Materials and Methods into soluble cytosolic (S) and membrane pellet (P) fractions followed by immunoprecipitation with antibodies to Lck, CAT or galectin-3, resolution by SDS-PAGE and fluorography. (B) Transfected cells were labelled with [^{3}H]palmitate and processed as for A. (b) A 7 day exposure and (c) a 36 day exposure of the same fluorograph. (C) as for A and B except that the cells were labelled with [^{3}H]myristate. Variation in the intensity of bands is due to different levels of expression in different transfections.

ing of the chimaeras into the S2 GEM fraction (Fig. 4A). This is particularly striking with LckN-Gal, in which case the dually acylated N terminus causes a dramatic redistribution from the S1 mainly to the S2 fraction of the extract (Fig. 4A).

Tritiated palmitate labeling experiments confirmed that the major proportion of the S-acylated CAT and galectin-3 (>80%) are located in GEMs (Fig. 4B). Mutation of the N-terminal cysteines greatly reduced the proportion of the proteins present in the GEM fraction but did not completely eliminate association, especially for the LckN-Gal protein (Fig. 4A). Thus S-acylation supplies much of the specificity for association with GEMs. By contrast non-S-acylated proteins seem to be located in the cytosolic fraction or non-specifically associated with intracellular membranes and GEM microdomains.

DISCUSSION

Caveolae and GEMs, both plasma membrane specialisations resistant to detergent solubilisation, are spatially adjacent domains strongly implicated in transmembrane signalling (Lisanti et al., 1994; Mineo et al., 1996; Song et al., 1996). In this manuscript we demonstrate that the N-terminal 10 amino acids of Lck are both necessary and sufficient to cause dual acylation of heterologous chimaeric proteins and to retarget them to cold non-ionic detergent-insoluble regions of the plasma membrane of transfected cells. Dual acylation has previously been shown to mediate tight membrane binding and association of Src family members with cold non-ionic detergent-insoluble membrane domains or GEMs (Alland et al.,

1994; Shenoy-Scaria et al., 1993, 1994; Kwong and Lublin, 1995; Rodgers et al., 1994). The relationship between these domains and caveolae has been controversial but these structures do not appear to be one and the same, although they both resist detergent extraction under similar conditions (Schnitzer et al., 1995; Kurzchalia et al., 1995). A myristoyl group in combination with a nearby polybasic stretch of amino acids can also encode plasma membrane localisation but does not cause proteins to become cold non-ionic detergent-insoluble (Shenoy-Scaria et al., 1993, 1994; Kwong and Lublin, 1995; Silverman and Resh, 1992). Lymphocytes and neuroblastoma cells have been shown to contain GEMs in the absence of caveolin and caveolae (Fra et al., 1994; Gorodinsky and Harris, 1995), and transfection of caveolin-1 into lymphocytes results in the de novo formation of caveolae (Fra et al., 1995). Purified caveolae have been reported to be enriched in caveolin and sphingolipid G_{M1}, non-receptor tyrosine kinases, heterotrimeric G-proteins and small GTP-binding proteins including Ras (Schnitzer et al., 1995; Song et al., 1996). GPI-anchored proteins, on the other hand, are not enriched in caveolae but are concentrated in detergent-resistant domains which are intimately associated with them (Schnitzer et al., 1995; Danielsen and van Deurs, 1995; Mayor et al., 1994). The proximity of these two domains may facilitate the coupling of GPI-anchored proteins to signal transduction systems (Lisanti et al., 1994). Although a useful operational method for identifying proteins partitioning into these two structures, cold non-ionic detergent resistance is not an absolute test of the localisation of a given protein to either

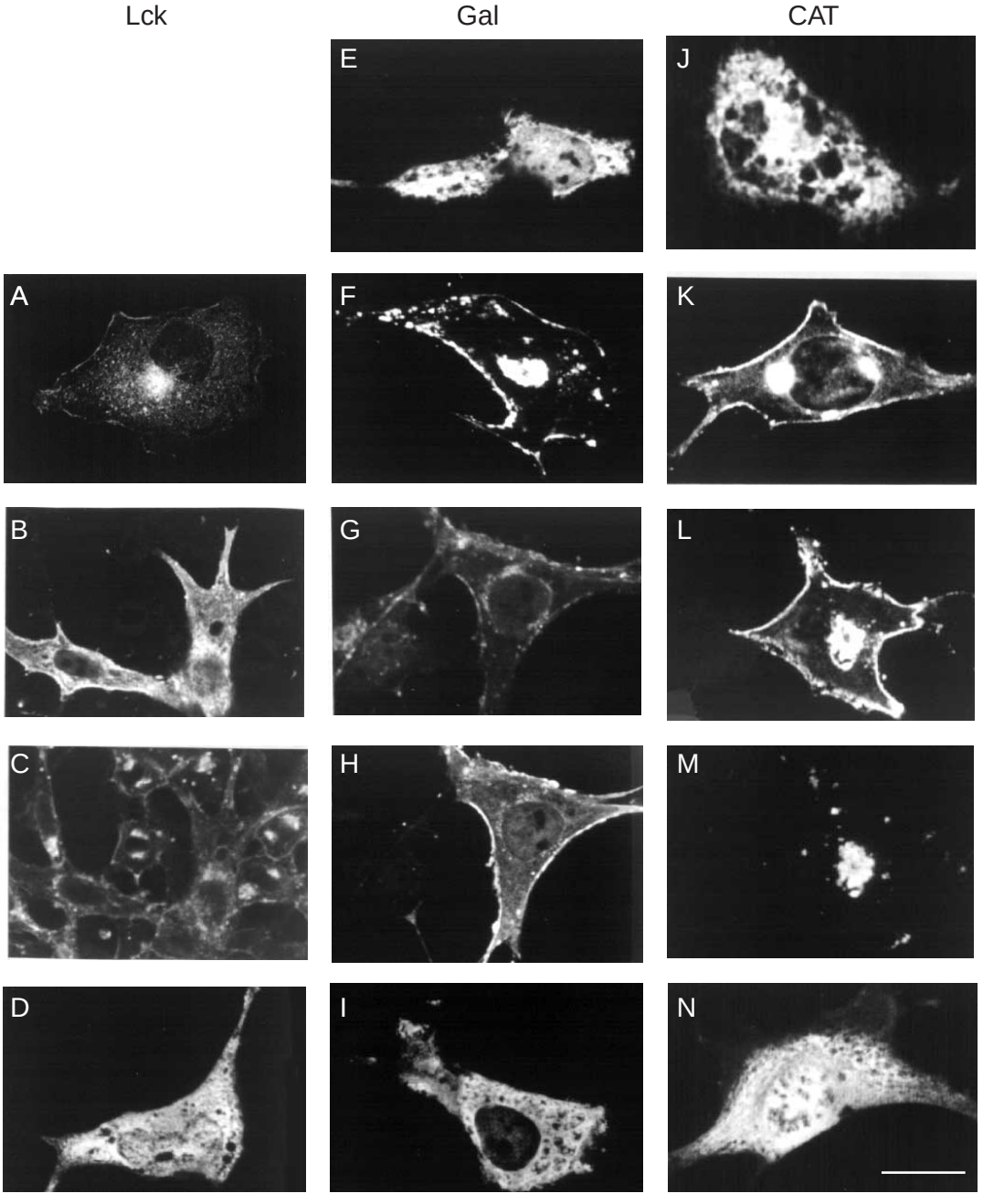

Fig. 3. Immunofluorescence confocal microscope localisation of Lck and chimaeric constructs. Cos cells expressing the constructs (see below) were stained with antibodies to Lck (A,B,D), galectin-3 (Gal; E,F,G,I), CAT (J,K,L,N) or with wheat germ agglutinin (WGA; C,H,M) and visualised by confocal microscopy. (B/C, G/H and L/M) Doubly stained to show the perinuclear co-distribution of Lck and WGA. Transfected constructs were: A,B,C, Lck; D, Lck(P^-); E, Gal; F,G,H, LckN-Gal; I, LckN(P^-)-Gal; J, CAT; K,L,M, LckN-CAT; N, LckN(P^-)-CAT.

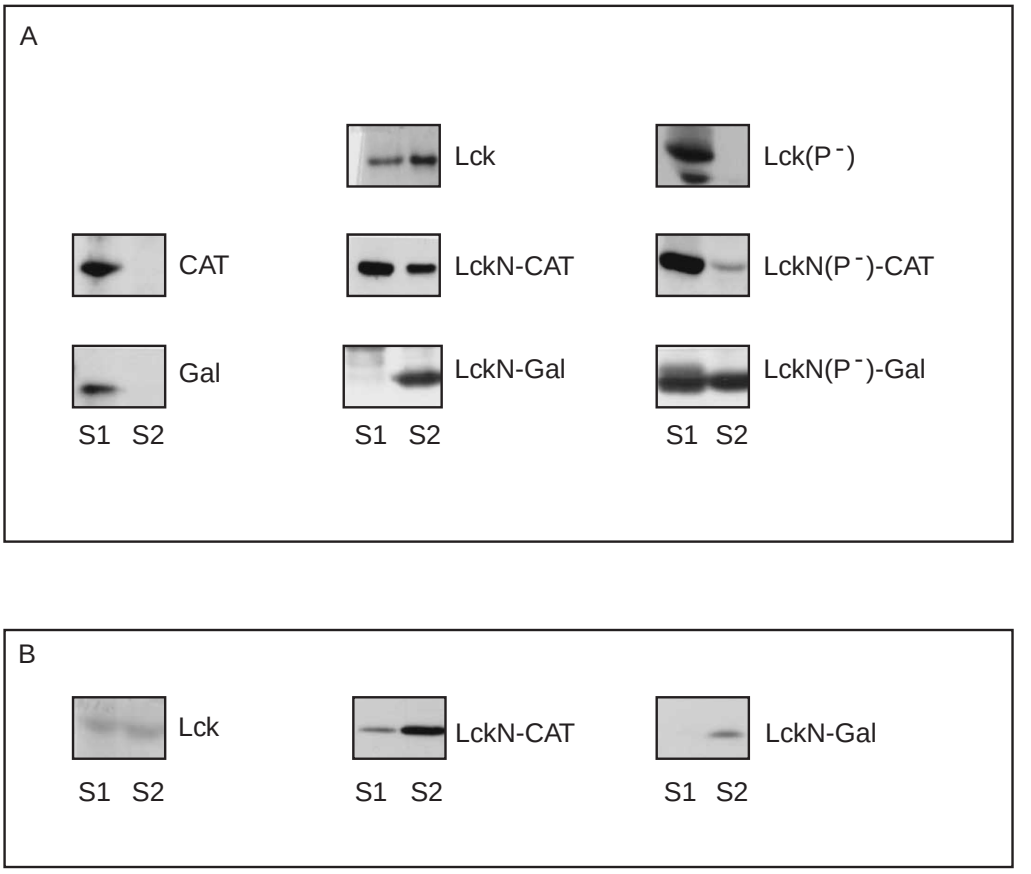

Fig. 4. Cold Triton X-100 partitioning of Lck and chimaeric constructs. Cos cells transfected to express the indicated constructs were labelled with Tran ^{35}S-label (A) or [^{3}H]palmitate (B) and extracted with Triton X-100 as described in Materials and Methods. S1, 4°C Triton X-100-soluble fraction; S2, 4°C Triton X-100-insoluble fraction, solubilised by Triton X-100 at 37°C (GEM fraction). Variation in the intensity of bands is due to different levels of expression in different transfections.

of them. H-Ras, which is readily extracted by non-ionic detergent, is nevertheless found associated with caveolae purified without the use of detergent (Song et al., 1996).

The MetGlyCys motifs of Src family members have been shown to be necessary and sufficient to specify dual acylation, membrane binding and partitioning into a cold non-ionic detergent-insoluble fraction in the context of a Src PTK using chimaeras of the N-terminal regions of Lck and Fyn with Src (Shenoy-Scaria et al., 1994). In addition, mutational analyses have revealed that the cysteine S-acylation sites are crucial for these properties (Shenoy-Scaria et al., 1993, 1994; Rodgers et al., 1994; Kwong and Lublin, 1995; Alland et al., 1994). However, other protein interaction domains (SH2, SH3 and kinase) in Src PTKs could contribute to localisation. Here we demonstrate that the lymphocyte-specific PTK Lck expressed in Cos cells shows an intracellular localisation at the plasma membrane and a perinuclear vesicular site indistinguishable from its distribution in T cells (Ley et al., 1994) suggesting that lymphocyte-specific proteins do not play an obligatory role in its localisation.The perinuclear Lck-positive compartment in T cells has been identified as an endosomal structure (Ley et al., 1994). We have not attempted rigorously to identify this structure in transfected Cos cells because the physiological relevance of this compartment in these cells to that in T cells is difficult to assess. However, the Lck-positive perinuclear compartment in Cos cells partly co-distributes with WGA, a marker which stains the Golgi complex. This need not imply that Lck is actually localised in the Golgi complex in these cells because the perinuclear region contains many membranous organelles. The in vivo reversibility of S-acylation of Lck (Paige et al., 1993) and other proteins (Magee et al., 1987; Milligan et al., 1995) could allow them to leave their initial location at the plasma membrane or within plasma membrane subdomains and translocate to another site, either as part of the signalling pathway or as a down-regulatory mechanism.

We chose two normally cytosolic proteins, CAT and galectin-3, as partners for our chimaeras. The Lck dual acylation motif targeted both of these to the plasma membrane albeit with different efficiencies. Little difference was seen when total galectin-3 and CAT were analysed (Fig. 2A) but the stoicheiometry of lipid modification is unknown. The extent of [^{3}H]myristate-labelled galectin-3 retargeting was similar to that of [^{3}H]myristate-labelled CAT (Fig. 2C), suggesting that myristoylation may be essentially complete in the transfected cells. Removal of S-acylation sites had a comparable effect on [^{3}H]myristate-labelled galectin-3 to that on [^{3}H]myristate-labelled CAT (Fig. 2C), suggesting that S-acylation played a major role in membrane association. The [^{3}H]palmitate-labelled proteins were essentially quantitatively associated with the membrane fraction (Fig. 2B) demonstrating that doubly acylated proteins have strong membrane-binding affinity. A proportion of both [^{3}H]myristate-labelled chimaeras is present in the soluble fraction, especially in the absence of S-acylation (Fig. 2C), confirming that myristoylation alone is not sufficient for strong membrane binding and suggesting that not all the myristoylated protein subsequently becomes S-acylated.

Finally, the ability of the Lck dual acylation motif to retarget different proteins to the plasma membrane and GEMs may prove a useful tool in studying the signalling mechanisms

operating at these specialised sites. An analogous approach has been used to retarget components of the Ras signalling cascade such as GAP (Huang et al., 1993), Sos (Quilliam et al., 1994) and Raf (Stokoe et al., 1994; Leevers et al., 1994) and to clarify the roles of these proteins within this pathway. It will be interesting to see whether dual acylation motifs with different numbers and spacings of lipid modifications, or other variations in sequence, can target proteins to membrane subdomains differing subtly in their composition and function.

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