S-acylation of LCK protein tyrosine kinase is essential for its signalling function in T lymphocytes

Panagiotis S.Kabouridis[1,2],
Anthony I.Magee[2,3] and Steven C.Ley[1,3]

Divisions of [1]Cellular Immunology and [2]Membrane Biology,
National Institute for Medical Research, The Ridgeway, Mill Hill,
London NW7 1AA, UK

[3]Corresponding authors

LCK is a non-receptor protein tyrosine kinase required for signal transduction via the T-cell antigen receptor (TCR). LCK N-terminus is *S*-acylated on Cys3 and Cys5, in addition to its myristoylation on Gly2. Here the role of *S*-acylation in LCK function was examined. Transient transfection of COS-18 cells, which express a CD8-ζ chimera on their surface, revealed that LCK mutants that were singly *S*-acylated were able to target to the plasma membrane and to phosphorylate CD8-ζ. A non-*S*-acylated LCK mutant did not target to the plasma membrane and failed to phosphorylate CD8-ζ, although it was catalytically active. Fusion of non-*S*-acylated LCK to a transmembrane protein, CD16:7, allowed its plasma membrane targeting and also phosphorylation of CD8-ζ when expressed in COS-18 cells. Thus *S*-acylation targets LCK to the plasma membrane where it can interact with the TCR. When expressed in LCK-negative JCam-1.6 T cells, delocalized, non-*S*-acylated LCK was completely non-functional. Singly *S*-acylated LCK mutants, which were expressed in part at the plasma membrane, efficiently reconstituted the induced association of phospho-ζ with ZAP-70 and intracellular Ca^{2+} fluxes triggered by the TCR. Induction of the late signalling proteins, CD69 and NFAT, was also reconstituted, although at reduced levels. The transmembrane LCK chimera also supported the induction of tyrosine phosphorylation and Ca^{2+} flux by the TCR in JCam-1.6 cells. However, induction of ERK MAP kinase was reduced and the chimera was incapable of reconstituting induced CD69 or NFAT expression. These data indicate that LCK must be attached to the plasma membrane via dual acylation of its N-terminus to function properly in TCR signalling.
Keywords: LCK/localization/*S*-acylation/signalling

Introduction

The induction of protein tyrosine kinase (PTK) activity by the T-cell antigen receptor (TCR) is essential to couple it to downstream pathways which trigger proliferation and differentiation of resting T cells into effector T cells (Weiss and Littman, 1994). However, the component subunits of the TCR do not contain any intrinsic tyrosine kinase domains. Rather, the TCR initiates signal transduc-tion by interacting sequentially with two different families of cytoplasmic non-receptor PTKs.

The first of these is the SRC family, in particular LCK and FYN (Perlmutter *et al.*, 1993). Analysis of LCK-deficient T-cell lines has indicated that LCK is absolutely required for TCR signalling (Karnitz *et al.*, 1992; Straus and Weiss, 1992). Indeed, in the absence of LCK, the TCR fails to induce any tyrosine phosphorylation of cytoplasmic proteins, and all downstream signalling events are blocked. Consistent with its important role in TCR signalling, T cell development is severely impaired in LCK null mice (Molina *et al.*, 1992). Genetic studies have also indicated that FYN is involved in TCR signalling in mature thymocytes (Appleby *et al.*, 1992; Stein *et al.*, 1992) and, in certain T-cell lines, may be associated with the TCR (Samelson *et al.*, 1990). However, from the phenotype of FYN null mice, it appears that the role of FYN in TCR signalling may be restricted to particular stages of T-cell development (Appleby *et al.*, 1992; Stein *et al.*, 1992).

The second family of PTKs which interact with the TCR comprises ZAP-70 (Chan *et al.*, 1992) and SYK (Taniguchi *et al.*, 1991). After stimulation, ZAP-70 and SYK are recruited to phosphorylated CD3 and ζ subunits of the TCR, where they are in turn tyrosine phosphorylated (Chan *et al.*, 1992, 1994b; Straus and Weiss, 1993; Wange *et al.*, 1993; Chan and Shaw, 1996). ZAP-70 and SYK bind to the TCR via their two N-terminal SH2 domains which interact with specific phosphorylated tyrosines of the cytoplasmic tails of the CD3 complex (γδε) and ζ dimers in immunoreceptor tyrosine-based activation motifs (ITAMs) (Chan *et al.*, 1994a; Chan and Shaw, 1996). In an LCK-deficient T cell line, TCR ITAMs are not phosphorylated and ZAP-70 is not recruited to the TCR after stimulation, indicating that ZAP-70 plays a role downstream of LCK (Straus and Weiss, 1992; Iwashima *et al.*, 1994). Studies in COS cells have suggested that one function of LCK is to phosphorylate ITAMs of the TCR, thereby mediating association of the receptor with ZAP-70 and its subsequent phosphorylation by LCK (Iwashima *et al.*, 1994; Chan *et al.*, 1995). Consistent with this hypothesis, both constitutive and inducible tyrosine phosphorylation of ζ is abolished in LCK null thymocytes (van Oers *et al.*, 1996). Tyrosine phosphorylation of ZAP-70 activates its kinase activity (Chan *et al.*, 1995; Wange *et al.*, 1995) and, together with LCK, mediates tyrosine phosphorylation of multiple intracellular proteins which trigger T-cell proliferation. An essential role for ZAP-70 in TCR signalling and T-cell development has been revealed by genetic studies in both murine and human systems (Hivroz and Fischer, 1994; Negishi *et al.*, 1995). In contrast, SYK does not appear to be essential for αβ T cell development (Turner *et al.*, 1995), although the development of epithelial γδ T cells is disrupted in SYK-

Table I. Schematic representation of LCK acylation mutants

		Myr ↓	Pal ↓		Pal ↓						
WT	Met	**Gly**	**Cys**	Val	**Cys**	Ser	Ser	Asn	Pro	Glu	Asp
C3A	Met	**Gly**	*Ala*	Val	**Cys**	Ser	Ser	Asn	Pro	Glu	Asp
C5A	Met	**Gly**	**Cys**	Val	*Ala*	Ser	Ser	Asn	Pro	Glu	Asp
C3,5A	Met	**Gly**	*Ala*	Val	*Ala*	Ser	Ser	Asn	Pro	Glu	Asp

The first 11 amino acids of murine LCK are shown in three letter code, with amino acids that are modified by myristoylation (Myr) or S-acylation (Pal) in bold type. Mutated amino acids are shown in italics.

negative mice (Mallick-Wood *et al.*, 1996). The precise role of SYK in signalling via the TCR is presently unclear. However, recent experiments have indicated that SYK has different activation requirements to ZAP-70 and does not require LCK expression (Chu *et al.*, 1996). Thus it is likely that ZAP-70 and SYK play distinct roles in TCR signalling.

Many proteins are now known to be post-translationally modified by the covalent addition of lipid moieties (Casey, 1995; Milligan *et al.*, 1995). Prominent among these are proteins involved in signalling via cell surface receptors, including the α and γ subunits of heterotrimeric G proteins (Milligan *et al.*, 1995), small GTP-binding proteins such as Ras (Newman and Magee, 1993) and the SRC family of protein tyrosine kinases (Resh, 1994). These lipid modifications have been found to play key roles in association of these otherwise hydrophilic proteins with the cytoplasmic face of specific cellular membranes. The N-terminal unique domain of LCK is modified by the addition of two different types of lipid (Shenoy-Scaria *et al.*, 1993; Koegl *et al.*, 1994; Rodgers *et al.*, 1994). Myristate, a saturated acyl group of 14 carbons, is added co-translationally to Gly2 via an amide bond, replacing the initiator methionine (Johnson *et al.*, 1994). Post-translationally, two longer chain fatty acyl groups, which are often C16 palmitates, are attached by labile thioester bonds to Cys3 and Cys5 (Milligan *et al.*, 1995). In this study, the role of S-acylation of LCK has been investigated by analysis of point mutants expressed in COS-18 cells and in a leukaemic T-cell line which does not express functional LCK. These experiments indicate that LCK S-acylation is required for it to couple the TCR to downstream signalling pathways which, in part, reflects its role in correct targeting of LCK to the plasma membrane.

Results

N-terminal S-acylation is required for cortical targeting of LCK

One of our laboratories has demonstrated previously that LCK is localized predominantly to the plasma membrane and also to peri-centrosomal vesicles in human T lymphocytes (Ley *et al.*, 1994a). The unique region of LCK is modified by the addition of a myristate group to Gly2 which is added co-translationally (Johnson *et al.*, 1994). Recent studies from this and other laboratories have indicated that the LCK unique region is also modified by the attachment of S-acyl groups to Cys3 and Cys5 (Shenoy-Scaria *et al.*, 1993; Koegl *et al.*, 1994; Rodgers *et al.*, 1994). To investigate the role of S-acylation of the LCK unique region in its localization and function, three point mutants were generated in which either Cys3 (C3A), Cys5

(C5A) or both cysteines (C3,5A) were substituted by an alanine (Table I). The C3A, C5A and C3,5A mutants are myristoylated but they lack one (C3A and C5A) or both (C3,5A) of the S-acyl attachment sites.

Localization of the LCK mutants was first analysed by transient expression in COS-18 cells and subcellular fractionation. Wild-type (WT) LCK was found exclusively in the particulate fraction (Figure 1A). The majority of the singly S-acylated C3A and C5A mutants, 71 and 92% respectively, was also found in the particulate fraction. In contrast, the minority of the C3,5A mutant was detected in the particulate fraction (33%). This crude analysis suggested that S-acylation of LCK was affecting its localization. This was analysed in more detail by immunofluorescence and confocal microscopy. In these experiments, transfected COS-18 cells were first made non-adherent and spherical by vigorous pipetting to facilitate detection of plasma membrane staining. WT LCK was detected exclusively at the plasma membrane, whereas the C3,5A mutant was found diffusely throughout the cytoplasm and also in the nucleus (Figure 1C). The C3A and C5A mutants showed an intermediate distribution and were detected both at the plasma membrane and in the cytoplasm.

The role of acylation in LCK localization in T cells was also investigated by stably expressing the panel of mutants in a derivative of the Jurkat T-cell line, JCam-1.6, which expresses low levels of a catalytically inactive deletion mutant of LCK (Straus and Weiss, 1992). Subcellular fractionation revealed that WT LCK was almost exclusively in the particulate fraction (93%), similar to COS-18 cells (Figure 1B). Removal of one or both S-acylation sites shifted the LCK into the soluble fraction. This shift was particularly pronounced with the C3A mutant where the majority of the protein (69%) was detected in the soluble fraction. Immunofluorescence and confocal microscopy demonstrated that the WT protein was localized to the plasma membrane and to perinuclear vesicles (Figure 1D), as shown previously (Ley *et al.*, 1994a). The C3A and C5A mutants were also localized to the plasma membrane, although a significant fraction was also detected diffusely distributed throughout the cytoplasm. In contrast, the C3,5A mutant was not detected at the plasma membrane but was present throughout the cytoplasm. Taken together, these data demonstrated that S-acylation was important in targeting LCK to the plasma membrane in both COS-18 and J-Cam-1.6 cells.

N-terminal S-acylation is not required for the enzymatic activity of LCK

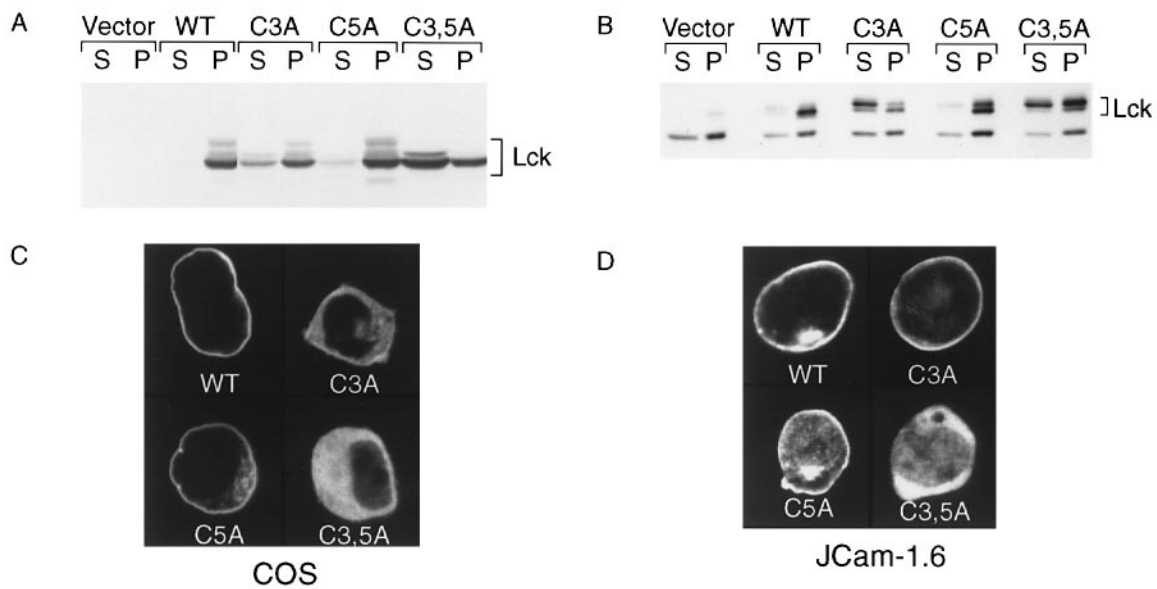Before analysing the signalling function of the LCK mutants, it was important to determine whether S-acylation

Fig. 1. Subcellular localization of LCK mutants expressed in COS-18 and JCam-1.6 T cells. COS-18 cells (**A** and **C**) were transiently transfected with the LCK cDNA constructs indicated. JCam-1.6 cells (**B** and **D**) were stably transfected with plasmids encoding each of the panel of LCK mutants. Clones were then isolated in which the level of transfected LCK was similar or greater than transfected WT LCK. (A and B) Cells were disrupted by freeze–thawing and homogenization and then separated into soluble (S) and particulate (P) fractions by ultracentrifugation. Equal aliquots from the two fractions were then resolved by 10% SDS–PAGE, transferred onto PVDF membrane and probed with anti-LCK antibodies. The band that represents the transfected LCK is shown in brackets. In (B) the lower band represents the truncated, non-functional LCK form present in JCam-1.6 cells. (C and D) Cells were fixed with paraformaldehyde, permeabilized and then stained for expression of LCK. The images shown are single confocal sections through representative transfected cells. Untransfected cells gave no detectable staining (data not shown).

was required for LCK enzymatic activity. To investigate this, expression vectors encoding each mutant were transfected into COS-18 cells which were then cultured for 48 h. Total cell lysates were then resolved by SDS–PAGE and probed for phosphotyrosyl (PTyr) proteins. Figure 2A indicates that expression of each of the LCK mutants resulted in induction of several PTyr proteins compared with empty vector control. The most prominent of these was a polypeptide of ~55 kDa which probably corresponded to transfected LCK itself. The C3,5A mutant, which was localized predominantly in the cytosol, was particularly active in the induction of PTyr proteins, many of which had mobilities distinct from those induced by the plasma membrane-targeted LCK mutants. These data indicated that there was no requirement for *S*-acylation for LCK enzymatic activity *in vivo*. In addition, these results suggested that the delocalized C3,5A LCK mutant was accessible to distinct substrates from both the WT protein and singly *S*-acylated mutants that were associated with the plasma membrane. To assay the activity of LCK directly, the various mutants were immunoprecipitated from transfected COS-18 cells and tested for their ability to phosphorylate RR-SRC peptide *in vitro*. The RR-SRC peptide corresponds to residues 111–122 of SRC and contains its auto-phosphorylation site (Wong and Goldberg, 1983). As shown in Figure 2B, all the *S*-acylation mutants had enzymatic activity comparable with the WT molecule.

The LCK mutants were also immunoprecipitated from the panel of stably transfected JCam-1.6 cell lines and assayed for their activity *in vitro*. To increase sensitivity in these assays, a different peptide, referred to as C-peptide, was used, which has been demonstrated to be the optimal substrate for LCK (Songyang and Cantley, 1995). All of

the mutants isolated from transfected J-Cam-1.6 T cells were found to have a specific activity similar to WT LCK protein, as was found with transfected COS-18 cells (Figure 2C).

A non-S-acylated LCK mutant cannot phosphorylate a CD8-ζ chimera expressed in COS-18 cells

COS-18 cells stably express on their surface a chimera comprising the extracellular and transmembrane portions of the human CD8α chain fused to the cytoplasmic domain of the TCR ζ chain (Iwashima *et al.*, 1994). Transient expression of WT LCK in COS-18 cells results in phosphorylation of the CD8-ζ chimera on tyrosines within ITAMs of the cytoplasmic tail of ζ. Co-expression of ZAP-70 with LCK results in association of ZAP-70 with phosphorylated ITAMs and its tyrosine phosphorylation. COS-18 cells, therefore, provide a simplified heterologous model to study sequential interactions between the TCR and the two non-receptor PTKs, LCK and ZAP-70.

As a first step to investigate the role of LCK *S*-acylation in its functional interaction with the TCR, COS-18 cells were transfected with cDNAs encoding the various LCK mutants with or without ZAP-70 cDNA. As shown in Figure 3A (left side), WT LCK and singly *S*-acylated C3A and C5A mutants were able to phosphorylate the CD8-ζ chimera. In contrast, the C3,5A mutant was unable to increase the tyrosine phosphorylation of the chimera above background levels although this mutant was expressed at levels comparable with WT LCK (Figure 3B). ZAP-70 associated with the CD8-ζ chimera, resulting in its increased tyrosine phosphorylation, when co-expressed with WT LCK or either of the two singly *S*-acylated mutants (Figure 3A, right side). The non-
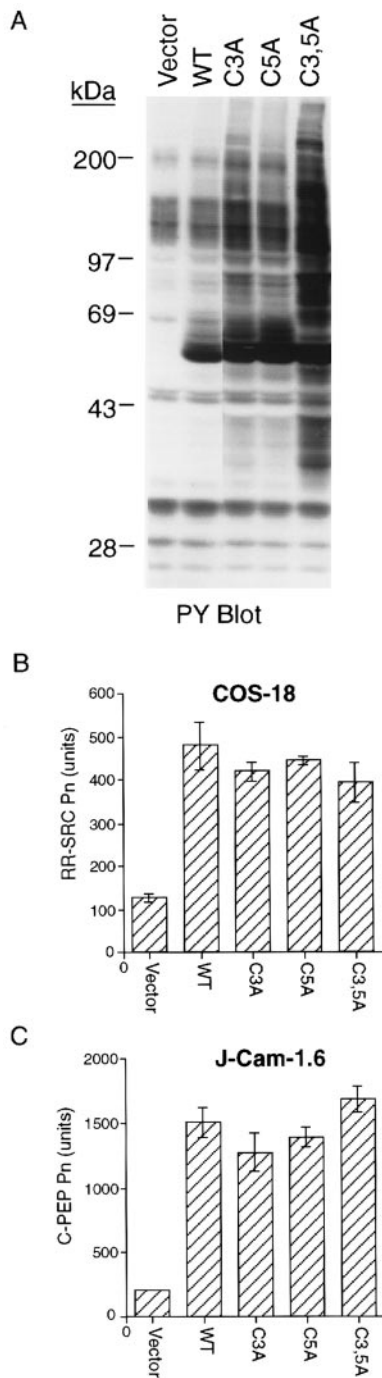
Fig. 2. LCK *S*-acylation mutants are enzymatically active both *in vivo* and *in vitro*. (**A**) Lysates were prepared from COS-18 cells transiently transfected with plasmids encoding the various LCK acylation mutants, as indicated. Proteins were resolved by SDS–PAGE, transferred onto PVDF membrane and immunoblotted with anti-PTyr mAb. The PVDF membrane was then stripped and reprobed with anti-LCK antiserum to confirm expression of the transfected LCK protein (not shown). (**B**) LCK mutants from transiently transfected COS-18 cells were immunoprecipitated and assayed for their ability to phosphorylate RR-SRC peptide *in vitro*. Results shown are normalized against the amount of LCK in immunoprecipitates as determined by immunoblotting, and are expressed in arbitrary units. Data are the mean ($\pm$ SE) of duplicate assays. (**C**) LCK was immunoprecipitated from JCam-1.6 clones expressing the indicated LCK mutants and assayed for its ability to phosphorylate C-peptide. Results are presented as in (B).
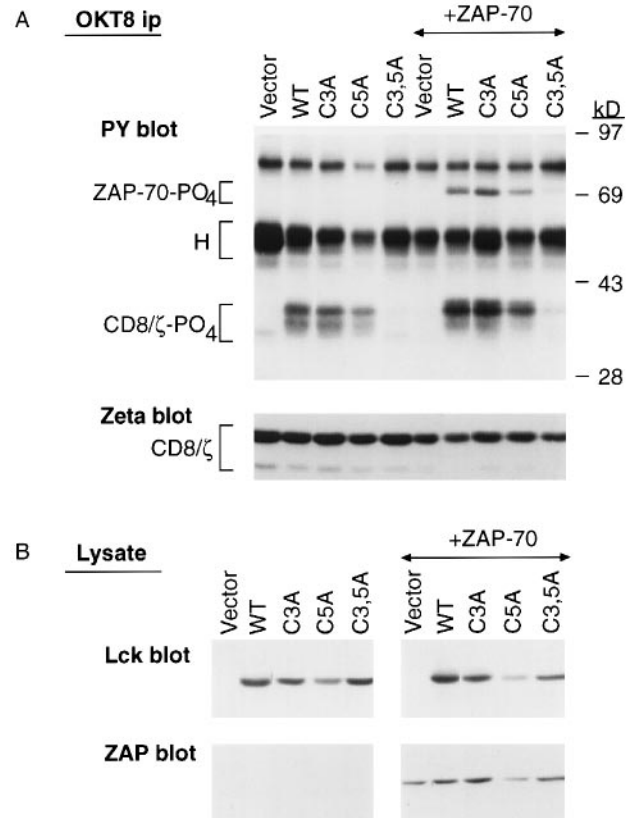
Fig. 3. Induction of CD8-ζ phosphorylation by LCK mutants in COS-18 cells. COS-18 cells were transiently transfected with vectors encoding the various LCK mutants in the presence and absence of ZAP-70 cDNA. (**A**) CD8-ζ was immunoprecipitated from cell lysates using the OKT8 mAb, resolved by 10% SDS–PAGE and transferred electrophoretically to PVDF membrane. The blot was then probed sequentially with an anti-PTyr mAb (PY) followed by an anti-ζ antibody (zeta). The positions of phospho-ZAP-70 (ZAP-70-PO$_4$) and phospho-CD8-ζ (CD8-ζ-PO$_4$) are indicated. H denotes the heavy chain of the immunoprecipitating antibody. (**B**) Equivalent amounts of total cell lysates from the transfected COS-18 cells used in (A) were resolved by SDS–PAGE. The expression of transfected LCK and ZAP-70 was then checked by sequential immunoblotting of the PVDF membrane with the appropriate antibodies.

S-acylated C3,5A mutant, however, was again unable to induce phosphorylation of CD8-ζ or its association with ZAP-70 in the doubly transfected cells. Western blot analysis demonstrated that this mutant and the co-transfected ZAP-70 were expressed at levels similar to that achieved in the WT LCK transfection (Figure 3B). In conclusion, these experiments indicated that non-*S*-acylated LCK, which did not stably interact with the plasma membrane, also failed to phosphorylate CD8-ζ when expressed in COS-18 cells.

Non-S-acylated LCK cannot reconstitute early signalling events triggered by the TCR in JCam-1.6 T cells

JCam-1.6 is a mutant derivative of the Jurkat T-cell line which expresses low levels of a mutant form of LCK that is catalytically inactive (Straus and Weiss, 1992). Stimulation of JCam-1.6 T cells with CD3 antibody fails to induce early or late signalling events triggered by the TCR. TCR signalling, however, may be reconstituted by transfection of JCam-1.6 cells with WT LCK. To investi-
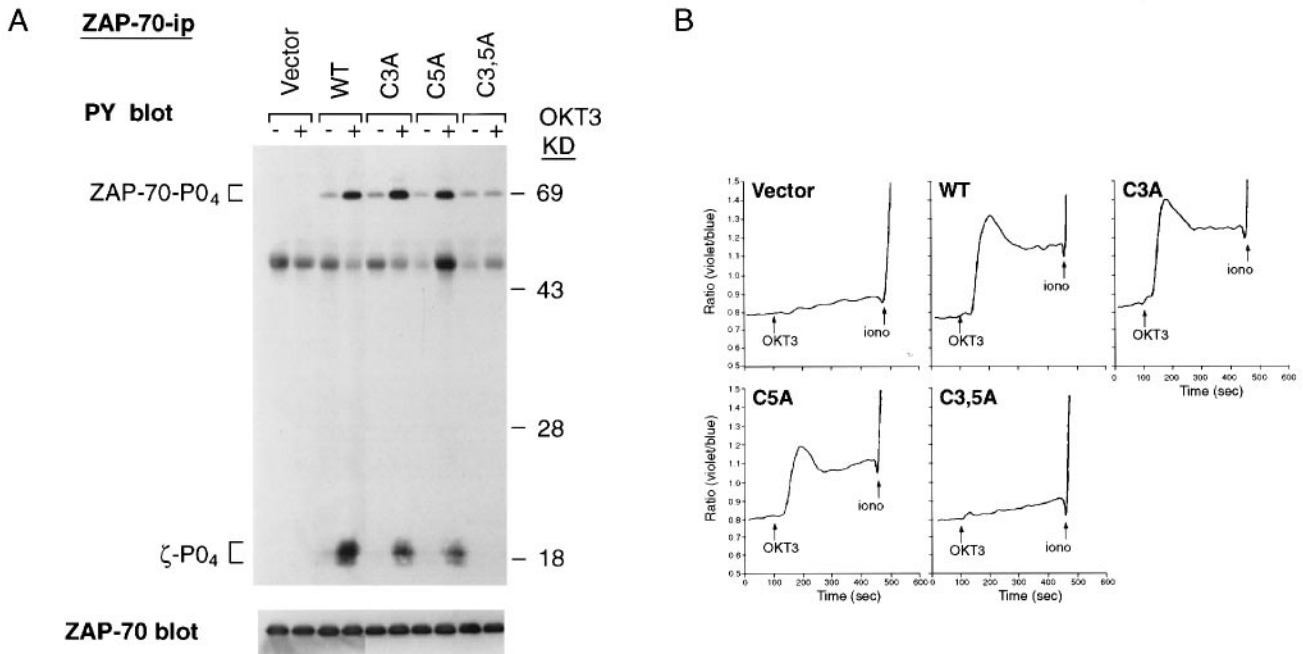
Fig. 4. Analysis of early signalling events triggered by the TCR in LCK-transfected JCam-1.6 cells. (**A**) Each of the clones was stimulated for 5 min with 1 μg/ml of F(ab')$_2$ fragments of the CD3 antibody OKT3 or left unstimulated and then lysed in 1% NP-40 lysis buffer. ZAP-70 was then isolated from cell lysates by immunoprecipitation and resolved by 12.5% SDS–PAGE. Tyrosine-phosphorylated proteins were detected by immunoblotting with an anti-PTyr mAb. The positions of phospho-ZAP-70 and phospho-ζ are indicated on the left of the panel. To check that equivalent levels of ZAP-70 were present in each immunoprecipitate, the PVDF membrane was stripped and probed with an anti-ZAP-70 antibody. Qualitatively similar results were obtained with at least two other clones for each of the LCK mutants (data not shown). (**B**) Transfected JCam-1.6 cells were loaded with the Ca^{2+}-interacting agent Indo-1 and then stimulated with OKT3 F(ab')$_2$ antibody. Changes in the the level of intracellular free Ca^{2+} are shown as a function of time. Successful loading with Indo-1 was confirmed by subsequently treating the cells with ionomycin. The times at which CD3 antibody and ionomycin were added are indicated.

gate the role of LCK acylation in signalling via the TCR in T cells, JCam-1.6 cells were transfected with various LCK mutants and stable clones expressing levels of transfected LCK similar to or greater than that of WT LCK were selected for analysis. For each LCK mutant, qualitatively similar results to those shown below were obtained with other clones tested (data not shown).

As discussed in the Introduction, TCR cross-linking rapidly induces LCK to phosphorylate ITAMs of the ζ chain (Weiss and Littman, 1994). ZAP-70 is then recruited to the TCR via binding of its two N-terminal SH2 domains and is itself tyrosine phosphorylated. To investigate the importance of LCK *S*-acylation in these early signalling events, the various JCam-1.6 clones were stimulated with F(ab)$_2$ fragments of the mitogenic CD3 monoclonal antibody OKT3 for 5 min and ZAP-70 protein was immunoprecipitated from cell lysates. Introduction of WT LCK or either of the singly *S*-acylated mutants into JCam-1.6 reconstituted the ability of the TCR to induce tyrosine phosphorylation of ZAP-70 and its association with the TCR (Figure 4A). In contrast, non-*S*-acylated C3,5A LCK failed to reconstitute inducible phosphorylation of ZAP-70 or its association with phospho-ζ, although this clone expressed high levels of transfected LCK.

Similarly to the results with ZAP-70 tyrosine phosphorylation, TCR cross-linking of cell lines expressing singly acylated LCK induced qualitatively similar patterns of total PTyr proteins to the WT LCK transfectant (data not shown). In contrast, the C3,5A LCK mutant failed to support any TCR-induced tyrosine phosphorylation,

although several bands were constitutively phosphorylated when compared with the empty vector control (data not shown). Thus, *S*-acylation of LCK was essential for the TCR to induce tyrosine phosphorylation of any intracellular proteins.

The panel of JCam-1.6 clones was also analysed for their ability to induce increases in intracellular free Ca^{2+} following TCR cross-linking. Both singly *S*-acylated LCK mutants induced Ca^{2+} levels similar to that achieved in the WT clone (Figure 4B). However, non-*S*-acylated C3,5A mutant failed to induce any increase in intracellular free Ca^{2+} following TCR cross-linking. Taken together, these data indicated that the LCK *S*-acylation mutants which were at least in part targeted to the plasma membrane were able to reconstitute early signalling events triggered by the TCR. In contrast, the non-*S*-acylated C3,5A mutant, which was completely delocalized, was non-functional.

LCK S-acylation mutants are defective in reconstituting late signalling events triggered by the TCR in JCam-1.6 cells

Late activation events following stimulation of the TCR include induction of the cell surface activation antigen, CD69 (Testi *et al.*, 1989), and of the T cell-specific transcription factor, NFAT, which is involved in transcriptional regulation of the interleukin-2 (IL-2) gene (Jain *et al.*, 1995). In order to investigate the ability of the various LCK mutants to support the first of these late activation events in JCam-1.6 cells, the panel of clones was stimulated with CD3 antibody for 48 h, following
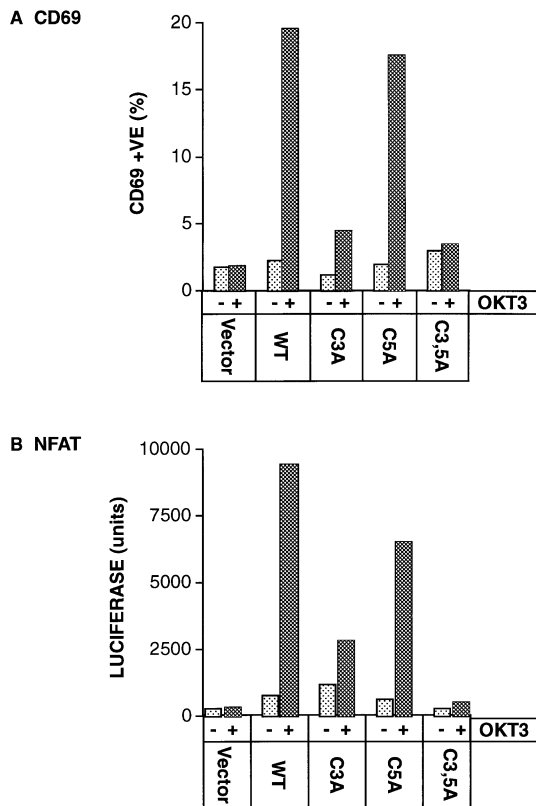
A CD69

B NFAT

Fig. 5. Analysis of late signalling events triggered by the TCR in LCK-transfected JCam-1.6 cells. (**A**) A total of 1×10^6 cells from each of the JCam-1.6 clones were cultured in the presence of 10 μg/ml OKT3 F(ab′)$_2$ antibody for 48 h. CD69 expression was then determined by immunofluorescence and flow cytometry. The percentage of cells which were CD69 positive was determined using an FITC-conjugated mouse myeloma to set the background levels of staining. Similar results were obtained in two other experiments. (**B**) LCK-expressing JCam-1.6 cells were transiently transfected with 15 μg of the NFAT–luciferase reporter construct, pBR322-3×NFAT-Luc. The cells were then cultured for 2 h and stimulated with 10 μg/ml OKT3 antibody (+) or left unstimulated (–). After a further 12 h in culture, cells were lysed and luciferase activity assayed. Data are presented in arbitrary units as a mean of duplicate measurements (± SE). Qualitatively similar results were obtained in three separate experiments.

which the cells were analysed for CD69 expression by immunofluorescence and flow cytometry. WT LCK induced expression of CD69 as expected (Figure 5A). Both singly *S*-acylated mutants were able to induce the expression of CD69, although the level of induced expression in C3A mutant-transfected cells was markedly reduced compared with WT LCK. The C3,5A mutant was completely unable to support the induction of CD69 expression following TCR stimulation.

In order to assay the induction of NFAT transcription factor, each JCam-1.6 clone was transfected with a construct containing the luciferase reporter gene under the control of three copies of the NFAT regulatory element (Verweij *et al.*, 1990). After 2 h in culture, cells were stimulated with CD3 antibody, or left unstimulated, and recultured for a further 12 h before harvesting. Cell lysates were then prepared and luciferase activity assayed. A similar pattern of responsiveness was seen as with CD69 expression. Thus WT LCK induced NFAT following CD3 cross-linking as expected. The singly *S*-acylated C5A

mutant was slightly impaired relative to WT in the induction of NFAT, whereas the C3A mutant induced only 30% of WT levels of NFAT following CD3 stimulation. The C3,5A mutant was essentially inactive. All transfected cell lines induced similar levels of NFAT expression in response to stimulation with Ca^{2+} ionophore and phorbol ester (data not shown).

In conclusion, the experiments in this section indicate that both singly *S*-acylated LCK mutants were able to reconstitute late signalling pathways triggered by the TCR. However, neither of these mutants was as effective as the WT LCK protein at inducing these late signalling events. This suggested that all three attached lipid groups were required for LCK to work with maximum efficiency in reconstituting TCR signalling in the JCam-1.6 cells. The non-*S*-acylated C3,5A mutant was not capable of coupling the TCR to late signalling events, as expected from its inability to induce early signalling events following TCR cross-linking.

Retargeting of non-S-acylated LCK to the plasma membrane reconstitutes early TCR-induced signalling events in JCam-1.6 cells

The experiments in both COS-18 cells and JCam-1.6 cells revealed a correlation between plasma membrane targeting of LCK mutants and their ability to interact functionally with the TCR. These results, therefore, suggested that one of the primary functions of LCK *S*-acylation is to target it to the plasma membrane. To investigate this hypothesis, the function was tested of LCK which could be expressed at the plasma membrane independently of its lipid modifications. A chimera was constructed by fusing the entire LCK coding sequence to the extracellular domain of CD16 and the transmembrane domain of CD7 to generate 16:7:LCK-WT, as shown in Figure 6A (Kolanus *et al.*, 1993; kindly provided by Brian Seed, Boston, MA). This protein contains intact *S*-acylation sites on LCK but cannot be myristoylated. A mutant of this chimera was generated by PCR in which the two *S*-acylation sites on LCK were mutated to generate the 16:7:LCK-C3,5A chimera. *In vivo* labelling with [^{3}H]palmitate confirmed that the 16:7:LCK-C3,5A chimera was not *S*-acylated, in contrast to 16:7:LCK-WT (data not shown). The functional experiments described below were all carried out on the non-acylated 16:7:LCK-C3,5A chimera. Similar results were obtained with 16:7:LCK-WT chimera (data not shown).

In initial experiments, cDNAs encoding WT LCK, LCK C3,5A and the 16:7:LCK-C3,5A chimera were transiently expressed in COS-18 cells and tested for their ability to phosphorylate CD8-ζ. As shown previously, WT LCK, but not the C3,5A mutant, could phosphorylate CD8-ζ. However, the 16:7:LCK-C3,5A chimera was able to induce high levels of tyrosine phosphorylation of CD8-ζ (Figure 6B). Thus retargeting to the plasma membrane overcame the acylation requirement for LCK to interact functionally with CD8-ζ when expressed in COS-18 cells. Co-transfection of ZAP-70 cDNA with 16:7:LCK-C3,5A cDNA also resulted in the association of ZAP-70 with CD8-ζ and its tyrosine phosphorylation (data not shown).

To study the function of 16:7:LCK-C3,5A in T cells, this cDNA construct was stably transfected into JCam-1.6 cells and an oligoclonal population of cells isolated, using fluorescence activated cell sorting (FACS), which
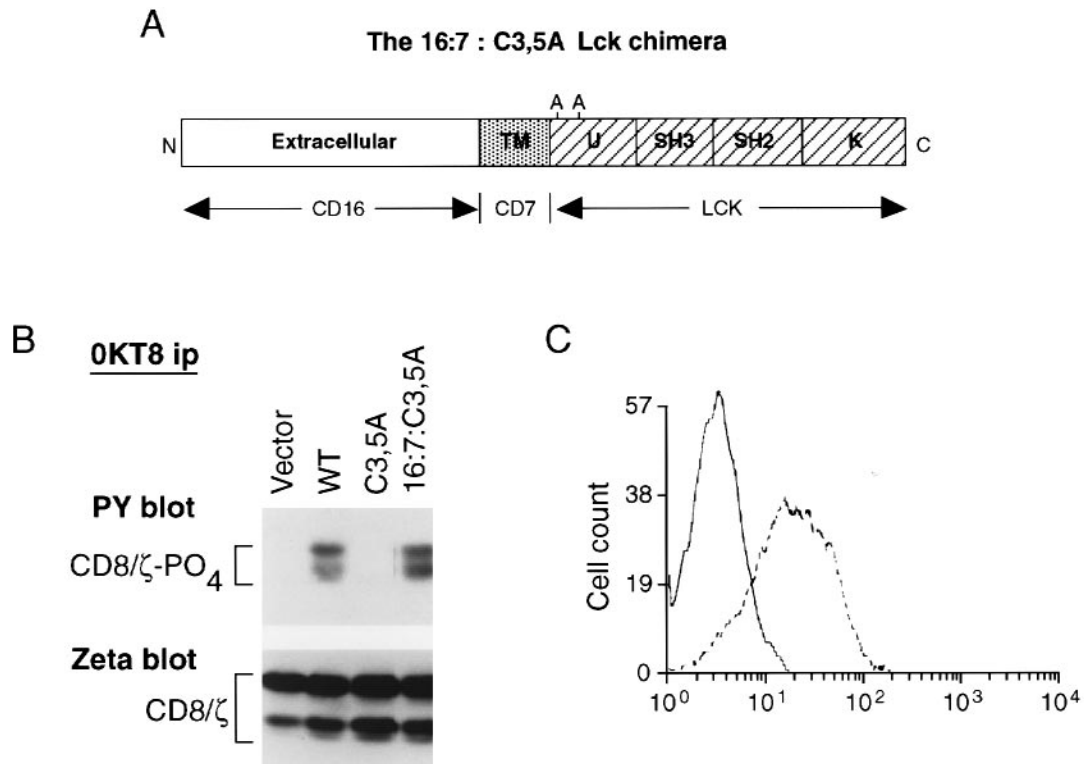
Fig. 6. Analysis of 16:7:LCK-C3,5A LCK chimera expressed in COS-18 and JCam-1.6 cells. (**A**) Schematic depiction of the 16:7:LCK-C3,5A chimera used in this study. (**B**) COS-18 cells were transfected with the indicated expression vectors and then CD8-ζ immunoprecipitated from cell lysates and immunoblotted sequentially for PTyr and ζ. The position of phospho-CD8-ζ is indicated on the left of the panel. Expression of transfected LCK or 16:7:LCK chimera was confirmed by immunoblotting of aliquots of total cell lysate used for immunoprecipitation, with an anti-LCK antibody (data not shown). (**C**) The expression level of the 16:7:C3,5A LCK chimera in stably transfected JCam-1.6 cells was determined by flow cytometry (dashed line). The background fluorescence was set with an FITC-conjugated anti-Ig antibody (solid line).
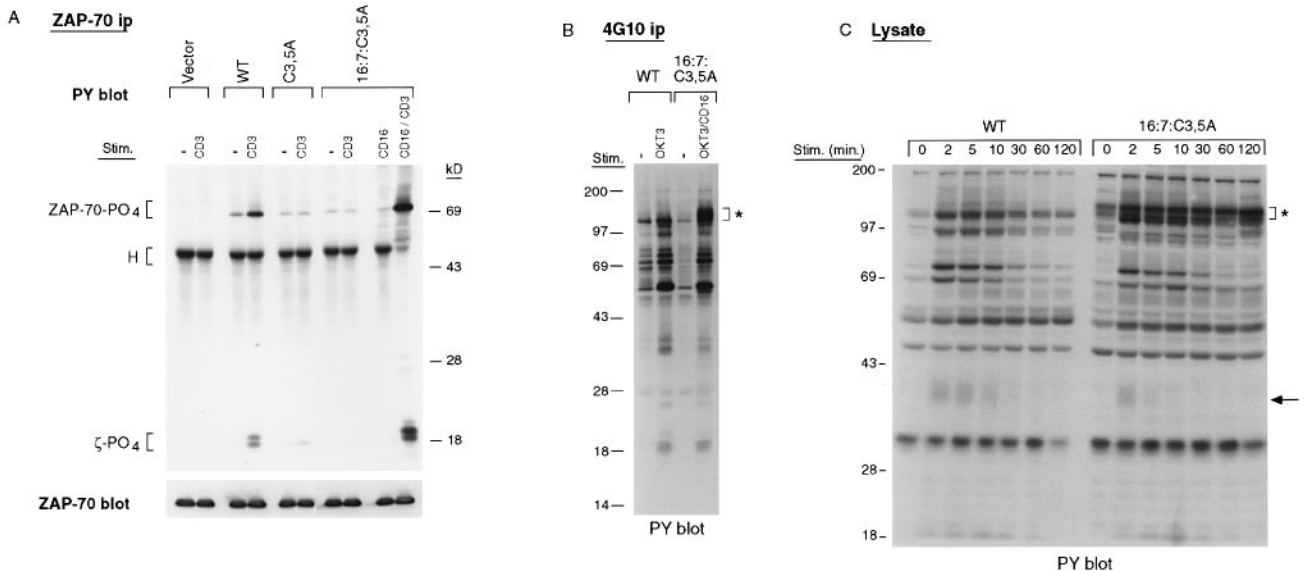

Fig. 7. Analysis of early tyrosine phosphorylation events triggered by the 16:7:LCK-C3,5A chimera in transfected JCam-1.6 cells. (**A**) JCam-1.6 cells stably expressing on their surface the 16:7:LCK-C3,5A chimera were stimulated with CD3 and/or CD16 antibodies in the presence of anti-IgG antibody for 5 min or left unstimulated. Control JCam-1.6 cells transfected with WT or C3,5A LCK were stimulated with CD3 plus anti-Ig antibodies. ZAP-70 was then immunoprecipitated from cell lysates and immune complexes were resolved by 12.5% SDS–PAGE and immunoblotted with anti-PTyr mAb (upper panel). The positions of phospho-ZAP-70 (ZAP-70-PO$_4$) and phospho-ζ (ζ-PO$_4$) are indicated on the left of the panels. Blots were re-probed with anti-ZAP-70 antibody to confirm that equivalent amounts of antigen were immunoprecipitated from each of the JCam-1.6 clones (lower panel). (**B**) A total of 10×10^6 cells of the WT- or 16:7:LCK-C3,5A-expressing JCam-1.6 cells were left untreated or were stimulated as in (A). Tyrosine-phosphorylated proteins were immunoprecipitated from cell lysates with the anti-PTyr mAb 4G10, resolved by SDS–PAGE electrophoresis and detected with Western blotting using anti-PTyr mAb. The asterisk indicates the migration distance of the 16:7: LCK-C3,5A chimera. (**C**) LCK WT- and 16:7:LCK-C3,5A-expressing JCam-1.6 cells were stimulated as in (A) for the indicated times, and cell lysates representing 0.5×10^6 cells were analysed by 10% SDS–PAGE and probed with anti-phosphotyrosine antibodies. The asterisk indicates the 16:7:LCK-C3,5A chimera while the arrow shows the activation-induced 36 kDa phosphoprotein.
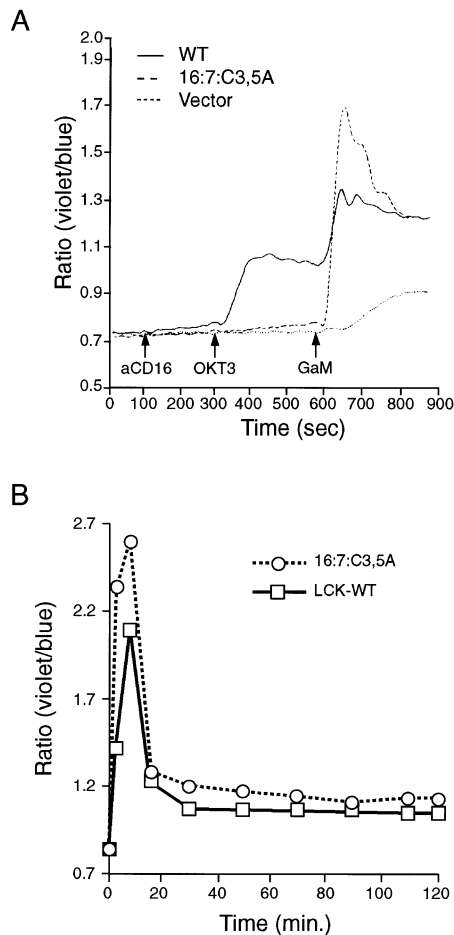
Fig. 8. Short and long term Ca^{2+} fluxes in WT- and 16:7:LCK-C3,5A-expressing cells. (**A**) The indicated transfected JCam-1.6 cells were loaded with Indo-1 and then sequentially stimulated with CD16 followed by CD3 and anti-IgG (goat anti-mouse) antibodies. Fluctuations in the levels of intracellular free Ca^{2+} before and after antibody stimulation are shown as a function of time. Successful loading with Indo-1 was confirmed by subsequently treating the cells with ionomycin (not shown). The times at which antibodies were added are indicated. (**B**) The WT- and 16:7:C3,5A-expressing clones were loaded with Indo-1 as in (A) and basal and stimulation-induced intracellular Ca^{2+} levels were monitored in a FACS. For each time point, ~1×10^4 cells were analysed and the violet/blue emission ratio was determined. All the values collected were plotted as the emission ratio versus time. This is one of two experiments performed with similar results.

Fig. 9. Analysis of late signalling events triggered by the 16:7:LCK-C3,5A chimera in transfected JCam-1.6 cells. (**A**) A total of 1×10^6 cells of each of the indicated JCam-1.6 clones were cultured with CD3 antibody, CD16 antibody, or both, as indicated, plus anti-IgG antibody for 48 h, or left unstimulated. The percentage of cells which expressed CD69 on their surface was then determined as in Figure 5. (**B**) Transfected JCam-1.6 cells were transiently transfected with 15 μg of the NFAT–luciferase reporter construct, pBR322-3×NFAT-Luc. The cells were then cultured for 2 h and stimulated with CD3 antibody, CD16 antibody, or both in the presence of anti-IgG antibody. After a further 12 h in culture, cells were lysed and luciferase activity assayed. Data are presented in arbitrary units as a mean of duplicate measurements (± SE).

showed high levels of surface CD16 staining (Figure 6C). Stimulation of the TCR on cells expressing 16:7:LCK-C3,5A failed to induce tyrosine phosphorylation of ZAP-70 or its association with the ζ chain (Figure 7A). In contrast, WT LCK induced both of these events as expected. However, if the chimera was stimulated with both CD3 and CD16 antibodies co-cross-linked with anti-Ig, ZAP-70 phosphorylation and association with the ζ chain was strongly induced. Control experiments demonstrated that addition of CD3 and CD16 antibodies in the absence of anti-Ig was insufficient to reconstitute signalling, suggesting that the chimera had to be brought into close proximity with the TCR in order to function (data not shown). The chimera was also able to reconstitute rapid increases in intracellular free Ca^{2+} when the TCR was co-cross-linked with CD16. This increase consistently was found to be greater than that achieved when JCam-
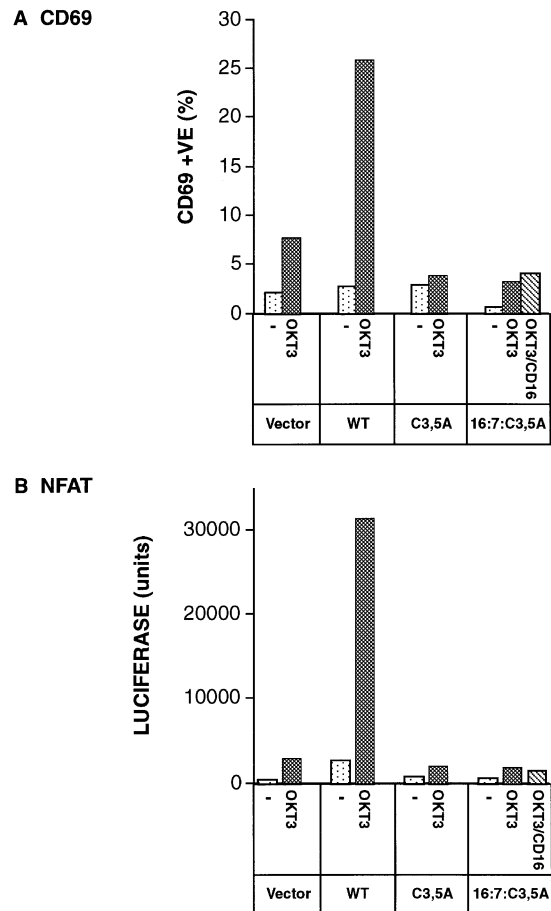
1.6 cells cells expressing WT LCK were stimulated with CD3 mAb and anti-Ig (Figure 8A). A kinetic experiment also demonstrated that the LCK chimera was able to sustain TCR-induced intracellular Ca^{2+} levels above basal levels for upto 2 h post-stimulation, similarly to WT LCK (Figure 8B). These results, therefore, suggested that there was no requirement for S-acylation of the plasma membrane retargeted LCK chimera to induce early signalling events triggered by the TCR. However, analysis of CD69 and NFAT induction revealed that the chimera was unable to induce these late activation events, even when the TCR and CD16 were co-aggregated on the cell surface (Figure 9A and B). In contrast, the WT LCK-expressing clone was able to induce both of these events, as expected. Thus the chimeric transmembrane form of LCK did not reconstitute TCR coupling to late signalling events in JCam-1.6 T cells.
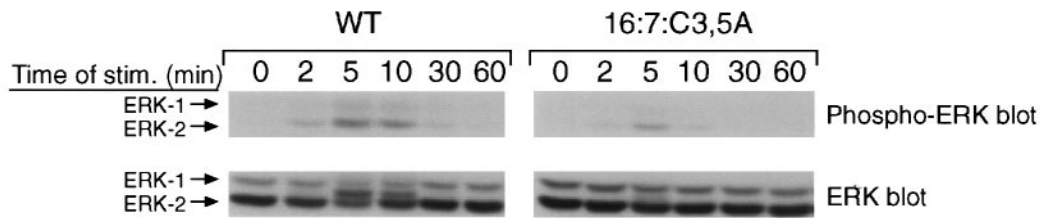
Fig. 10. Kinetics of ERK activation following stimulation of WT- and 16:7:LCK-C3,5A-expressing JCam-1.6 cells. WT- and 16:7:LCK-C3,5A-expressing JCam-1.6 cells were left untreated or were stimulated with OKT3 or OKT3 plus CD16 mAbs respectively, followed by cross-linking with anti-Ig antibody for the indicated times, and the phosphorylation of ERK-1 and ERK-2, in equal cell lysate aliquots, was detected by immunoblotting with anti-phospho-ERK antibody (upper panel). The same membrane was stripped and reprobed with anti-ERK-1/2 antiserum (lower panel). The migrating distances for ERK-1 and ERK-2 are indicated with arrows. The slower migrating species observed in the lower panel are indicative of ERK activation.

The 16:7:LCK-C3,5A chimera is deficient in its ability to reconstitute TCR-induced ERK activation in JCam-1.6 cells

The failure of the 16:7:LCK-C3,5A chimera to reconstitute late signalling events triggered by the TCR was not due to a quantitative reduction in the level of phosphorylation of ZAP-70 or its association with phospho-ζ (Figure 7A). To investigate whether the chimera was able to reconstitute the tyrosine phosphorylation of other intracellular proteins after TCR cross-linking, JCam-1.6 cells transfected with WT LCK or 16:7:LCK-C3,5A were stimulated with the indicated antibodies and PTyr proteins immunoprecipitated and Western blotted with an anti-PTyr monoclonal antibody (mAb). In Figure 7B, it can be seen that the pattern of PTyr bands induced by the TCR was very similar for JCam-1.6 cells transfected with either LCK construct. Thus the 16:7:LCK-C3,5A chimera appeared to facilitate the phosphorylation of all the major TCR-inducible PTyr proteins. A time-course experiment also indicated that the kinetics of TCR-induced phosphorylation of most of the major PTyr proteins in both cell lines were similar (Figure 7C). However, the phosphorylation of a 36 kDa PTyr protein (shown with an arrow) was found consistently to be more transient in cells expressing the 16:7:LCK-C3,5A chimera compared with cells reconstituted with WT LCK.

Activation of the Ras-MAP kinase pathway is required for induction of CD69 and NFAT by the TCR (D'Ambrosio *et al.*, 1994; Genot *et al.*, 1996). To investigate the possibility that ERK MAP kinase activation might be affected in the 16:7:LCK-C3,5A chimera-transfected JCam-1.6 cells, total cell lysates were prepared from cells stimulated for the indicated times with anti-CD3 mAb and electroblotted. Blots were then probed with a phospho-specific anti-ERK-1/2 antibody which recognizes the activated, tyrosine-phosphorylated forms of ERKs 1 and 2. The blot was then stripped and reprobed with an anti-ERK-1/2 antibody to detect the total amount of ERKs 1 and 2 in each lane. In Figure 10, it can be seen that activation of ERKs 1 and 2 was deficient in the 16:7:LCK-C3,5A chimera-transfected JCam-1.6 cells compared with the WT LCK transfectant. These data raised the possibility that inability of the LCK chimera to reconstitute late signalling events triggered by the TCR might result from its inability to support the efficient activation of ERK MAP kinases.

The 16:7:LCK-C3,5A chimera associates with ZAP-70 after TCR stimulation

TCR stimulation induces LCK to associate with ZAP-70 via the former protein's SH2 domain (Duplay *et al.*, 1994).
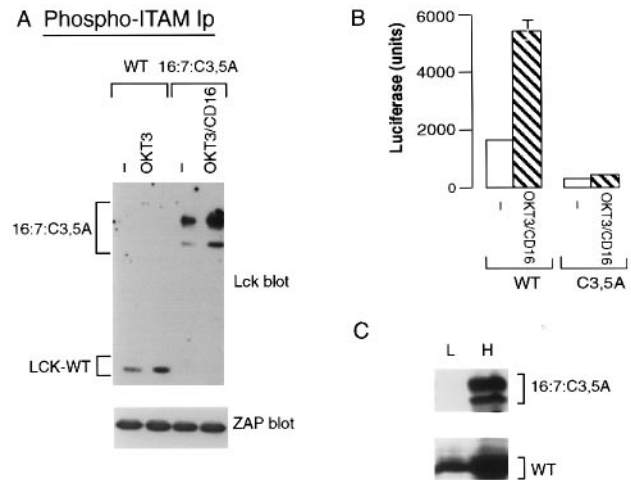

Fig. 11. The LCK chimera can interact with ZAP-70 and, in 16:7:LCK-C3,5A-expressing cells, co-expression of WT but not C3,5A LCK, can support TCR-induced NFAT production. Also the submembrane topology of the 16:7:C3,5A chimera differs from that of WT LCK. (**A**) Cells (30×10⁶) expressing WT or 16:7:C3,5A were stimulated with OKT3 or OKT3/CD16 mAb respectively followed by cross-linking with anti-mouse Ig, for 5 min. Following stimulation, ZAP-70 was precipitated from cell lysates using Affigel-coupled phospho-ITAM1 peptides. Immune complexes were resolved in 10% SDS–PAGE, transferred onto PVDF membrane, and co-immunoprecipitated LCK was detected with specific antibodies (upper panel). The same membrane was stripped and reprobed with ZAP-70-reacting antibodies (lower panel). The experiment shown is representative of four experiments done with similar results. (**B**) A total of 20×10⁶ 16:7:LCK-C3,5A-expressing cells were co-transfected with 15 μg of the NFAT–luciferase construct plus 25 μg of WT- or C3,5A LCK-containing plasmids as indicated. Activation of the cells and luciferase assays were performed as in Figure 9. The experiment shown is one of four performed with identical results. Treatment of the transfected cells with ionophore and phorbol ester induced comparable levels of NFAT-driven luciferase activity. (**C**) JCam-1.6 cells (50×10⁶) expressing WT or 16:7:LCK-C3,5A were lysed in 1% Triton X-100-containing buffer and the cell lysates were fractionated through a sucrose gradient as described in Materials and methods. The content of LCK in equal aliquots from high (H) and low (L) density sucrose fractions was assessed by Western analysis. The blot was intentionally overexposed in order to detect even minimal presence of LCK in the low density fraction.

It has been suggested that this interaction may be necessary for the functional cooperation between these PTKs during TCR signalling (Duplay *et al.*, 1994; Straus *et al.*, 1996). It was possible that the failure of 16:7:LCK-C3,5A to reconstitute CD69 and NFAT expression induced by the TCR in the JCam-1.6 cells might have resulted from its inability to form a complex with ZAP-70. To investigate this possibility, lysates were prepared from JCam-1.6 cells,

expressing either WT LCK or 16:7:LCK-C3,5A, with and without mAb stimulation of their TCRs. ZAP-70 was then isolated by incubating lysates with a synthetic phosphorylated oligopeptide, corresponding to the membrane-proximal ITAM of the ζ chain (Osman *et al.*, 1995), coupled to Affi-Gel 10 beads (Bio-Rad). The phospho-ITAM peptide interacted with ZAP-70 via its SH2 domains (Weiss and Littman, 1994), and, as a consequence, isolated ZAP-70 was not bound to phospho-ITAMs of the TCR. This method of purification of ZAP-70, therefore, avoided the possibility of isolating the 16:7:LCK-C3,5A chimera artefactually via its association with the TCR induced by the co-cross-linked CD3 and CD16 mAbs used for stimulating the cells. In Figure 11A, it can be seen that both WT LCK and the 16:7:LCK-C3,5A chimera inducibly associated with ZAP-70 after stimulation of the TCR. Thus the failure of 16:7:LCK-C3,5A to fully complement TCR signalling in the JCam-1.6 did not result from its failure to interact with ZAP-70.

Expression of cytosolic C3,5A LCK does not complement the signalling defect of the 16:7:LCK-C3,5A chimera

The *S*-acyl moieties on LCK turn over with a half-life that is much shorter than the half-life of the protein (Paige *et al.*, 1995). Deacylation may allow LCK to detach from the membrane after activation (Milligan *et al.*, 1995). Thus the failure of the 16:7:LCK-C3,5A chimera to support the induction of late activation events might have resulted from a requirement for detachment of LCK from the plasma membrane after TCR stimulation. This could not occur when LCK was anchored artificially via a transmembrane domain, as was the case for 16:7:LCK-C3,5A.

To investigate the possibility that LCK might need to detach from the plasma membrane to carry out its signalling functions, 16:7:LCK-C3,5A-expressing cells were transiently transfected with cDNAs encoding either WT or C3,5A LCK together with the NFAT luciferase reporter construct. The cells were then stimulated with co-cross-linked CD3 and CD16 mAbs, or left unstimulated, and NFAT-driven luciferase production was assayed after 12 h in culture. Transfection of WT LCK restored TCR induction of NFAT in the 16:7:LCK-C3,5A-expressing JCam-1.6 cells. Thus the TCR signalling pathways leading to NFAT production were intact in the JCam-1.6 cells which expressed 16:7:LCK-C3,5A. However, TCR induction of NFAT was not restored in cells transfected with cytosolic C3,5A LCK (Figure 11B). These data, therefore, did not suggest that the signalling defect of 16:7:LCK-C3,5A was due to its inability to detach from the plasma membrane after TCR stimulation.

The 16:7:LCK-C3,5A chimera is excluded from glycolipid-enriched microdomains

The plasma membrane is specialized into microdomains which are enriched in glycosphingolipids, sphingomyelin and cholesterol but depleted of phospholipids (Brown and Rose, 1992; Parton and Simons, 1995). These microdomains have been termed glycolipid-enriched membranes (GEMs) and are characterized by their insolubility in cold non-ionic detergents (Rodgers *et al.*, 1994). Some members of the SRC family of PTKs, including LCK, are associated with GEMs (Shenoy-Scaria *et al.*, 1993, 1994; Rodgers *et al.*, 1994). GEMs also contain glycosylphosphatidinositol (GPI)-linked proteins (Mayor *et al.*, 1994; Rodgers *et al.*, 1994) and heterotrimeric G proteins (Sargiacomo *et al.*, 1993). The association of LCK with both GPI-anchored proteins and with GEMs is dependent on its *S*-acylation (Shenoy-Scaria *et al.*, 1993; Rodgers *et al.*, 1994). Thus GEMs may be specialized microdomains that are involved in coupling GPI-linked receptors to activation of PTK activity.

It was possible that the 16:7:LCK-C3,5A chimera, although it was targeted to the plasma membrane, might not be accessible to GEMs. To investigate this possibility, cell lysates were prepared from WT- and 16:7:LCK-C3,5A-transfected JCam-1.6 cells. These lysates were then resolved on a discontinuous sucrose gradient by centrifugation and low density (30/5% sucrose interface) and high density (40% sucrose) fractions collected. GEMs partition into the low density fraction (Brown and Rose, 1992). Fractionated lysates were then probed for LCK; as expected WT LCK was clearly detected in the low density GEM fraction (Figure 11C). However, even after long exposure, none of the 16:7:LCK-C3,5A chimera was detected in the GEM fraction. These data, therefore, suggested that, within the plane of the plasma membrane, the 16:7:LCK-C3,5A chimera was not targeted identically to the WT LCK protein.

Discussion

This study demonstrates that *S*-acylation of the unique region of LCK is essential for its targeting to the plasma membrane. Myristoylation of LCK on its own was insufficient to attach LCK firmly to the plasma membrane, as revealed by analysis of the non-*S*-acylated C3,5A LCK mutant which was localized throughout the cytoplasm (Figure 1). This is consistent with experiments analysing the binding of myristoylated peptides to phospholipid vesicles, which indicate that myristoylation of a protein cannot stably bind it to a lipid bilayer (Peitzsch and McLaughlin, 1993; Bhatnagar and Gordon, 1997). To achieve detectable binding of LCK to membranes, *S*-acylation was required in addition to myristoylation, as indicated by the partial localization of singly *S*-acylated LCK to the plasma membrane. However, analysis of the distribution of WT LCK indicated that the attachment of two *S*-acyl groups and a myristate group was necessary to achieve high levels of membrane binding. The difference in localization of WT LCK and the two singly *S*-acylated mutants also suggests that most molecules of the WT protein carry three attached acyl groups.

In a separate series of experiments, this laboratory recently has demonstrated that the addition of the first 10 amino acids of LCK to two different soluble cytoplasmic proteins was sufficient to retarget them to the plasma membrane and also to vesicles next to the nucleus in COS-7 cells (Zlatkine *et al.*, 1997). Taken together with the data in this study, this suggests that the SH2 and SH3 domains of LCK are not essential for intracellular targeting of the fully lipid-modified protein. Rather, this is achieved by dual acylation of its first five amino acids with myristate and *S*-acyl moieties which directly bind LCK to the plasma membrane and perinuclear vesicles.

LCK is associated with the co-receptors CD4 and CD8 through their cytoplasmic domains and cysteine residues in the N-terminal unique domain of LCK, which are distinct from the sites of *S*-acylation (Rudd, 1990). A previous study demonstrated that a non-*S*-acylated C3S,5K mutant of LCK is not able to form a complex with CD4 or CD8 α when expressed in COS-7 cells, whereas singly *S*-acylated LCK mutants are able to complex with these surface molecules (Turner *et al.*, 1990). Thus, the binding of the N-terminal unique region of LCK to CD4 or CD8 α correlates with the targeting of LCK to the plasma membrane. In addition, the COS-18 and JCam-1.6 cells used in this study do not express CD4 or CD8. These data are consistent with the hypothesis that it is the *S*-acylation of LCK that targets it to the plasma membrane rather than interaction of its unique domain with cell surface CD4 or CD8 α.

One of the primary functions of LCK in TCR signalling is to phosphorylate ITAMs in the cytoplasmic tails of the CD3 complex and ζ homodimers (Weiss and Littman, 1994; Chan and Shaw, 1996). This facilitates recruitment of ZAP-70 PTK to the TCR, and ZAP-70 is then phosphorylated by LCK and activated. Two model cell lines, COS-18 (Iwashima *et al.*, 1994) and JCam-1.6 (Straus and Weiss, 1992), were used to study the role of LCK *S*-acylation in these early signalling events. Experiments with the heterologous COS-18 cell system indicated that phosphorylation of the CD8-ζ chimera and its association with ZAP-70 occurred only with LCK mutants which could localize, at least in part, to the plasma membrane (Figure 3). The delocalized C3,5A mutant was highly catalytically active (Figure 2B) and induced much higher levels of non-specific tyrosine phosphorylation than the correctly localized proteins (Figure 2A), but did not phosphorylate CD8-ζ (Figure 3). The failure of this mutant to phosphorylate the chimera was probably due to its inaccessibility to the ITAMs of the ζ cytoplasmic tail at the plasma membrane. Consistent with this hypothesis, the CD16.7:LCK-C3,5A chimera, which was neither myristoylated nor *S*-acylated on LCK but which was expressed efficiently at the plasma membrane, could induce tyrosine phosphorylation of CD8-ζ when expressed in COS-18 cells (Figure 6B). This suggests that one of the functions of *S*-acylation is to target LCK to the plasma membrane where it is in close proximity to the TCR.

Qualitatively similar results were obtained in the JCam-1.6 clones in which the function of LCK acylation mutants could be analysed in response to TCR ligation. Thus singly *S*-acylated LCK mutants, which were present at the plasma membrane, were able to interact functionally with the TCR to induce ζ phosphorylation and its subsequent association with ZAP-70 after stimulation with CD3 antibody (Figure 4). In contrast, the delocalized C3,5A mutant completely failed to reconstitute TCR signalling. The catalytic activity of this mutant was similar to WT LCK (Figure 2C), suggesting that its failure to complement the JCam-1.6 signalling defect was due to inability to interact with the TCR at the plasma membrane. The non-acylated 16:7:LCK-C3,5A chimera was able to induce efficiently both ζ phosphorylation and its association with ZAP-70 after co-cross-linking of the chimera with the TCR (Figure 7A). This again supports the hypothesis that a primary role of LCK *S*-acylation is to target it to the

plasma membrane. Steric hindrance by the extracellular domain of the 16:7:LCK-C3,5A chimera may explain why it was necessary to co-cross-link it with the TCR to reconstitute signalling in JCam-1.6 cells. The reconstitution of TCR-induced increases in intracellular free Ca^{2+} in response to TCR ligation in JCam-1.6 cells was also only achieved by LCK mutants which were present at the plasma membrane, and the delocalized C3,5A mutant was completely inactive (Figure 8A). Furthermore, the 16:7:LCK-C3,5A chimera could only induce increased intracellular free Ca^{2+} under conditions in which phospho-ζ was formed and associated with phospho-ZAP-70 after co-cross-linking. Thus ζ and ZAP-70 phosphorylation was closely coupled to subsequent increases in intracellular free Ca^{2+}.

The Sefton laboratory has also investigated the role of lipidation in the biological activity of a constitutively active mutant of LCK, LCK-Y505F (Yurchak and Sefton, 1995). In contrast to the data from this study, a non-*S*-acylated C3,5S mutant of LCK-Y505F was found to be catalytically inactive when expressed in 208F fibroblasts. The inactivity of this delocalized LCK-Y505F mutant correlates with its failure to be phosphorylated on Tyr394 (Yurchak *et al.*, 1996). Based on these data, this group have suggested that LCK must be associated with membranes in order to be catalytically active, perhaps by facilitating Tyr394 phosphorylation. However, the present study shows that the non-membrane-targeted C3,5A LCK mutant was highly biologically active when expressed in either COS-18 cells or JCam-1.6 cells. Similarly, a non-myristoylated cytoplasmic form of LCK is highly active when expressed in Sf9 insect cells (Carrera *et al.*, 1991). Taken together, these data suggest that membrane attachment is only required for LCK catalytic activity in certain cell types. This may reflect differences in the expression of either kinases or phosphatases that act on LCK to alter its basal state of phosphorylation, thereby changing its activity.

All of the LCK acylation mutants were found to be deficient, to different extents, in their ability to reconstitute TCR induction of two late events in JCam-1.6 cells, namely CD69 and NFAT expression (Figure 5). As expected, the C3,5A mutant, which did not reconstitute early tyrosine phosphorylation stimulated by the TCR in JCam-1.6 cells, also failed to support the induction of either CD69 or NFAT. The C3A mutant was able to support the induction of CD69 and NFAT expression after TCR stimulation, but to much lower levels than the WT protein. The C5A mutant was also slightly impaired in both of these late responses compared with WT. The difference in function between the two singly *S*-acylated mutants and WT protein was probably due to quantitative differences in the amount of LCK stably associated with the plasma membrane, although the total amount of each mutant LCK expressed was similar to WT. This correlates well with the relative efficiency of *S*-acylation, which this laboratory and that of Lublin have found to be higher for the C5A mutant than the C3A mutant (Koegl *et al.*, 1994; Kwong and Lublin, 1995). These data, however, contrast with those of Rodgers *et al.* (1994) who identified C5 as the major *S*-acylation site on LCK. Both of the singly *S*-acylated mutants could induce phospho-ζ and its association with phospho-ZAP-70 to levels comparable with WT (Figure 4). The phosphorylation of the major TCR-induced

PTyr proteins in JCam-1.6 cells transfected with the singly S-acylated mutants was also similar to WT LCK (data not shown). The induction of CD69 and NFAT, therefore, was not closely linked with the rapid tyrosine phosphorylations induced by TCR stimulation, and implies that LCK played an additional role in the activation process which was sensitive to its S-acylation status. However, it is possible that the TCR-induced phosphorylation of minor PTyr substrates, which were not evident when total PTyr proteins were analysed, was reduced with the singly S-acylated mutants, due to their inefficient targeting to the plasma membrane. This possibility is being investigated currently by two-dimensional gel electrophoresis.

Yurkchak and Sefton (1995) have also investigated the importance of LCK S-acylation in T-cell function. These investigators tested the ability of LCK-Y505F S-acylation mutants to induce IL-2 in stably transfected T-cell hybridomas in an antigen receptor-independent fashion. These experiments failed to detect any differences between WT LCK-Y505F and singly S-acylated mutants in the induction of IL-2, and concluded that S-acylation of either Cys3 or Cys5 was sufficient for full functional activity of LCK. These data contrast with the results in this study in which both of the singly S-acylated LCK mutants were functionally compromised relative to WT in the induction of either NFAT or CD69 following TCR stimulation (Figure 5). This difference probably arises from the use by Sefton and colleagues of mutants which had high levels of constitutive activity, which were effectively uncoupled from upstream regulatory events, with the result that the assay in T-hybridoma cells did not require TCR stimulation to induce IL-2. In contrast, this study investigated the function of LCK acylation mutants that were not mutationally activated in a T-cell line in which the induction of IL-2 is completely dependent on TCR stimulation and LCK activity. The requirements for S-acylation in LCK function, therefore, were necessarily more stringent and also more physiologically relevant. Indeed, it is not clear how LCK-Y505F induces IL-2 in T hybridomas, and whether this requires ζ and ZAP-70 phosphorylation.

Unlike the situation with the singly S-acylated LCK mutants, the function of the LCK transmembrane chimera was not limited by its level of expression at the plasma membrane (Figure 6C). Tyrosine phosphorylation of ZAP-70, TCR ζ and other intracellular proteins following TCR stimulation was very similar between the JCam-1.6 cells transfected with WT LCK or the 16:7:LCK-C3,5A chimera (Figure 7). The chimeric protein was also able to reconstitute TCR-induced increases in intracellular free Ca^{2+} to levels greater than the WT protein (Figure 8A). Thus retargeting of the LCK-C3,5A mutant to the plasma membrane by fusing it to a transmembrane protein restored some of its signalling functions. This is consistent with the hypothesis that the primary role of S-acylation is to target LCK to the plasma membrane where it can interact with the TCR. However, unlike the WT protein, the 16:7:LCK-C3,5A chimera was completely unable to reconstitute the induction of CD69 and NFAT by the TCR (Figure 9). The 16:7:LCK-C3,5A chimera was able to reconstitute a sustained increase in intracellular free Ca^{2+} in the JCam-1.6 cells (Figure 8B), which is necessary for TCR induction of IL-2 (Goldsmith and Weiss, 1988). However, ERK MAP kinase activation in JCam-1.6 cells

transfected with the chimera was reduced relative to the WT protein (Figure 10). Since the Ras-ERK pathway is required for induction of CD69 and NFAT by the TCR (D'Ambrosio et al., 1994; Genot et al., 1996), it is possible that deficient ERK activation by the chimera accounts for its inability to support these late activation events. Interestingly, anergic $CD4^+$ T cells are also deficient in their ability to activate the Ras-ERK pathway and to produce IL-2 following TCR stimulation (Fields et al., 1996; Li et al., 1996). The data in this study raise the possibility that an alteration of LCK function may contribute to the anergic phenotype which uncouples the TCR from the efficient activation of ERK MAP kinase.

Two different hypotheses may explain the partial complementation of the JCam-1.6 signalling defect when LCK was artificially expressed as a transmembrane protein. First, it was possible that LCK must detach from the plasma membrane after TCR stimulation, as a consequence of its S-deacylation. This might be important either as a mechanism to inactivate LCK or to give it access to cytosolic substrates (Milligan et al., 1995; Paige et al., 1995). This obviously could not occur when LCK was anchored via a transmembrane domain. However, co-transfection of the cytosolic C3,5A LCK mutant failed to reconstitute TCR-induced NFAT production in JCam-1.6 cells expressing the chimera (Figure 11B). Similarly, an activated C3,5A LCK-Y505F mutant also did not reconstitute TCR induction of NFAT in this cell line (data not shown). In contrast, WT LCK was able to restore normal TCR signalling (Figure 11B), confirming that the TCR signalling machinery was still intact in this cell line. Taken together, these data did not support the hypothesis that the signalling deficiency of the 16:7:LCK-C3,5A chimera resulted from its inability to detach from the plasma membrane after TCR stimulation.

A second explanation to account for the signalling deficiency of 16:7:LCK-C3,5A was its exclusion from GEMs, in contrast to the WT protein. Thus the LCK chimera was differentially distributed within the plane of the plasma membrane relative to the WT LCK protein. This may have affected the accessibility of LCK to critical target proteins which were located in the GEMs. Perhaps significantly, two recent studies have suggested that Ras is localized to caveolae, that share many properties in common with GEMs (Schnitzer et al., 1995; Mineo et al., 1996; Song et al., 1996). However, since lymphocytes do not contain caveolae but do contain GEMs (Fra et al., 1994), the relevant plasma membrane microdomain in JCam-1.6 cells must be the latter. The exclusion of the LCK chimera from GEMs, therefore, may prevent access to critical substrates which are involved in the regulation of Ras. A 36 kDa protein has been described that interacts with GRB2 via its SH2 domain in T lymphocytes after TCR stimulation (Buday et al., 1994; Sieh et al., 1994) and has been postulated to couple the TCR to the activation of Ras. Interestingly, kinetic experiments revealed that a 36 kDa PTyr protein was transiently phosphorylated in 16:7:LCK-C3,5A-expressing cells after TCR ligation, compared with a more sustained phosphorylation in cells expressing the WT protein (Figure 7C). Preliminary experiments have indicated that this 36 kDa PTyr protein can be precipitated with a GST–GRB2 fusion protein (data not shown). Thus it is possible that the 16:7:LCK-

C3,5A chimera is not able to sustain prolonged phosphorylation of the GRB2-associated 36 kDa PTyr protein and, as a consequence, Ras is not activated properly. This possibility currently is being investigated.

In conclusion, these data demonstrate an essential role for N-terminal *S*-acylation in the function of LCK in TCR signalling. This in part reflects a requirement for *S*-acylation to target LCK to the plasma membrane where it can interact with one of its critical substrates, the TCR. However, the inability of the 16.7:LCK-C,3,5A chimera to complement fully the signalling defect in the JCam-1.6 cells indicates that the attachment of LCK to the plasma membrane via dual acylation of its unique domain plays some additional role which is essential for it to carry out its function in TCR signalling.

Materials and methods

Generation of LCK constructs

A cDNA encoding wild-type mouse LCK (WT LCK) was subcloned into the *Kpn*I–*Xba*I sites of the pcDNA3^{neo} expression vector (Invitrogen). The LCK mutants C3A, C5A and C3,5A were generated from this WT cDNA using PCR with the following oligonucleotides as primers: 5′-CAGGTACCATGGGCGCTGTCTGCAGCTCA-3′ (C3A), 5′-CAGGTACCATGGGCTGTGTCGCCAGCTCA-3′ (C5A) and 5′-CAGGTACCATGGGCGCTGTCGCCAGCTCA-3′ (C3,5A). The oligonucleotide 5′-GTCGAAGTCTCTGACCGACAG-3′, containing the *Bam*HI restriction site at position 443 of the LCK coding region, was used as the reverse primer in the PCR reaction to amplify 450 bp fragments. The various PCR products were digested with *Kpn*I–*Bam*HI restriction enzymes and then inserted into the *Kpn*I–*Bam*HI-treated pcDNA3/LCK construct.

To generate the 16:7:LCK-C3,5A chimera, the oligonucleotide 5′-AAGCTTACGCGTATGGGCGCTGTCGCCAGC-3′ was used as a forward primer. This oligonucleotide, which contained an *Mlu*I restriction site 5′ of the initiation ATG codon, was used for PCR in combination with the reverse primer described above. Following amplification, the PCR products were digested with *Mlu*I and *Bam*HI restriction enzymes. The 16:7 fragment of the chimera was obtained from the 16:7:ZAP chimera (Kolanus *et al.*, 1993) following digestion with *Hin*dIII and *Mlu*I. The two DNA fragments were then used in a three way ligation with a *Hin*dIII–*Bam*HI-digested pcDNA3/LCK construct to generate the 16:7:LCK-C3,5A chimera sub-cloned into the pcDNA3^{neo} expression vector. All PCR products were verified by DNA sequencing.

The various LCK mutant and chimera constructs subcloned into the pcDNA3^{neo} expression vector were used for transient expression experiments in COS-18 cells. To generate stable JCam-1.6 cell lines, the cDNA constructs were subcloned into the pREP3 episomal expression vector (Hambor *et al.*, 1988).

Cell culture and transfections

COS-18 cell cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. The JCam-1.6 variant of E6.1 Jurkat cells was cultured in RPMI 1640 medium supplemented with 5% FCS and the above concentrations of L-glutamine and antibiotics. Both cell lines were maintained in a rapid growth phase prior to transfection.

For transient transfections, COS-18 cells were expanded to 75% confluence in 175 cm^2 culture flasks (Nunclon) and harvested by trypsinization. Detached cells were washed twice with HEPES-buffered saline (HBS: 140 mM NaCl, 5 mM KCl, 0.7 mM Na$_2$HPO$_4$, 20 mM HEPES pH 7.5). Approximately 5×10^6 cells, resuspended in 800 μl of HBS, were transferred into a 0.4 cm cuvette (Bio-Rad), and the indicated amount of the appropriate pcDNA3^{neo} expression vector DNA was added together with sonicated salmon sperm DNA (Promega) to give a total of 100 μg of DNA per transfection. Cells were transfected by electroporation (Flowgen electroporator; 350 V, 300 μF) and then cultured for 48 h in a 100×15 cm Petri dish (Nunclon) before harvesting.

For the generation of stable transfectants, JCam-1.6 cells were washed twice with FCS-free RPMI 1640 medium, and 10×10^6 cells were resuspended in 800 μl of the fresh serum-free medium and transferred into an electroporation cuvette (Bio-Rad). Twenty five μg of the appropriate pREP3 DNA construct was then added and the cell suspension electroporated (Bio-Rad Gene pulser; 330 V/960 μF). The cells were then cultured in 10 ml of complete medium for 24 h, hygromycin B was added to a final concentration of 0.25 mg/ml and the cells from a single transfection seeded into four 96-well plates at limiting dilution (<30% of wells giving rise to cell growth). Half of the medium from each well was replaced every 3 days with fresh medium plus hygromycin B, and 3 weeks later hygromycin B-resistant clones were assayed for LCK expression. Clones which expressed similar amounts of the transfected LCK protein were selected for further study.

Antibodies

The LCK-1 anti-LCK antibody was used for confocal microscopy and immunoprecipitations (Koegl *et al.*, 1994). For immunoblotting, the 2166 rabbit anti-LCK antibody was used, which was raised against a denatured GST–LCK fusion protein (from Sheldon Ratnovsky, BASF, Worcester, MA). The anti-CD8 mAb, OKT8, was a kind gift from Dr C.Tsoukas (San Diego, CA). The anti-CD3 antibody, OKT3, was obtained from the American Type Culture Collection (Rockville, MD), and (Fab′)$_2$ fragments of this antibody were kindly prepared by A.Tutt and M.Glennie (Tenovus, Southampton, UK). Tyrosine-phosphorylated proteins were detected using the 4G10 anti-PTyr mAb (from Brian Druker, Oregon Health Sciences University, Portland, OR). The ZAP-4 anti-ZAP-70 antiserum has been described previously (Huby *et al.*, 1995) and was affinity purified using the immunizing peptide for immunoblotting. The TCR ζ chain was recognized in Western blots using the N39 antiserum kindly provided by Jaime Sancho (Granada, Spain). The anti-CD16 mAb and the fluorescein isothiocyanate (FITC)-conjugated anti-CD69 mAb were both obtained from PharMingen. Activated ERK-1/2 was detected using the PhosphoPlus p44/42 MAPK antibody from New England Biolabs. A rabbit antiserum which recognizes both ERKs 1 and 2 in Western blots was provided by Jeremy Tavare (University of Bristol, UK).

Immunoprecipitation and Western blotting analysis

Transfected COS-18 cells were washed free of serum with phosphate-buffered saline (PBS) and then lysed with 1 ml of ice-cold immunoprecipitation buffer [IPB; 150 mM NaCl, 20 mM Tris–HCl pH 7.4, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM Na$_3$VO$_4$, 5 mM NaF and 5 μg/ml each of chymostatin, leupeptin and pepstatin] with scraping. JCam-1.6 cells stably expressing the various forms of the LCK protein were resuspended to 2×10^7 cells/ml in ice-cold RPMI medium, and were left unstimulated or were stimulated for 5 min with 1 μg/ml of OKT3 (Fab′)$_2$ fragments at 37°C. Cells were then pelleted by pulsing in a microcentrifuge and lysed in 1 ml of IPB for 15 min at 4°C. Cell lysates were cleared of insoluble debris by centrifugation at 13 000 *g* for 15 min at 4°C and then pre-cleared once by incubation with 10 μl of protein A–Sepharose (Pharmacia) for 15 min at 4°C. For immunoprecipitation, 10 μg of purified monoclonal antibody or 5–10 μl of antiserum were coupled covalently to 10 μl of protein A–Sepharose with dimethylpimelidate (Schneider *et al.*, 1982) and incubated with pre-cleared cell lysate for 4 h or overnight. Precipitation of ZAP-70 using a phospho-ITAM1 peptide coupled to Affigel 10 (Bio-Rad) was carried out as described previously (Osman *et al.*, 1995). Following extensive washing with ice-cold IPB, isolated protein was resolved by SDS–PAGE and transferred onto polyvinylidene difluoride (PVDF) membranes. Western blotting was carried out as described previously (Ley *et al.*, 1994b). PVDF membranes were stripped of bound antibody using the Amersham ECL protocol in experiments in which blots were probed for multiple antigens.

NFAT–luciferase assay

JCam-1.6 cells (10×10^6) expressing the various LCK mutants were transfected by electroporation with 15 μg of pBR322-3×NFAT-Luc vector (from Gerry Crabtree, Stanford, CA) using the methodology outlined above. Cells were then cultured at 37°C for 2 h and 10 μg of OKT3 antibody added to half of the transfected cell cultures. The remaining cultures were left unstimulated. After a further 12 h in culture, cells were lysed with 150 μl of lysis buffer [0.5% NP-40, 100 mM HK$_2$PO$_4$, 1 mM dithiothreitol (DTT) pH 7.8] for 10 min on ice. Post-nuclear supernatants were assayed for luciferase using the Promega luciferase assay kit with a Clinilumat (Berthold) luminometer. Assays were performed in duplicate and the results shown are the mean ± SE.

Calcium analysis

JCam-1.6 cells (5×10^6), transfected with the indicated LCK mutants, were incubated in 1 ml of complete medium supplemented with 3 mM of the Ca^{2+} indicator Indo-1 at 37°C for 1 h. Following incubation, the

cells were washed twice with LOCKS buffer (150 mM NaCl, 1 mM CaCl₂, 1 mM MgSO₄, 5 mM KCl, 10 mM glycine, 15 mM HEPES pH 7.4), resuspended in the same buffer to a concentration of 1×10^6 cells/ml and then kept on ice, protected from light, until analysis. Before analysis, the cells were pre-warmed in a 37°C waterbath for 1 min and Ca^{2+} fluctuations before and after the addition of the indicated ligands were monitored using an LS50 Perkin Elmer Luminescence spectrometer. Cells were excited at 355 nm and emission measured at 480 and 405 nm, representing free versus Ca^{2+}-associated Indo-1, to give an absorbance ratio. For prolonged Ca^{2+} measurements, cells were loaded with Indo-1 as described above with the only difference that cells were washed and then cultured in a 5% FCS-containing RPMI medium. Ca^{2+} levels before and after the addition of stimulating mAbs were determined with a Becton Dickinson FACS Vantage. For the indicated time points, a sample of $\sim 1 \times 10^4$ cells was analysed and the violet/blue emission ratio was determined. When not analysed in the FACS, cell cultures were kept in a 37°C waterbath.

Flow cytometric analysis of cell surface antigens

To determine cell surface expression of the 16:7:LCK-C3,5A chimera, immunofluorescent staining was performed as described previously (Ley *et al.*, 1994a) and cells were analysed on a Becton Dickinson FACS Vantage. To assay for the induction of CD69 expression, 1×10^6 cells of the appropriate LCK-transfected JCam-1.6 clone were cultured in a 24-well plate (Nunclon) in the presence or absence of 10 μg/ml of the CD3 mAb OKT3 for 48 h. In the case of the 16:7:C3,5A chimera, stimulation was performed with CD3 plus CD16 mAbs cross-linked with the addition of anti-Ig antibody. The cells were then stained with an FITC-conjugated CD69 mAb. Background fluorescence levels were set using an FITC-conjugated mouse IgG myeloma.

In vitro kinase assay for LCK activity

COS-18 cells were washed twice with PBS and then lysed in 1 ml of of ice cold buffer (SB) comprising 50 mM *N*-octyl-β-D-glucopyranoside (Sigma), 150 mM NaCl, 25 mM Tris, 1 mM Na₃VO₄, 2 mM Na₄P₂O₇, 20 mM NaF and 1 μg/ml each of chymostatin, leupeptin and pepstatin, pH 7.2. Cell lysates were cleared of insoluble material by centrifugation at 13 000 *g* for 10 min. Precipitation with anti-LCK antibody coupled to protein A–Sepharose for 4 h was performed as described above. Immunoprecipitates were then washed five times in lysis buffer and once in kinase buffer (100 mM NaCl, 50 mM HEPES pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂ and 1 mM Na₃VO₄). Each immunoprecipitate was then resuspended in 20 μl of kinase buffer supplemented with 10 μM ATP, 5 μCi [³²P]ATP and 1 mM RR-SRC peptide substrate (Gibco-BRL) and incubated at room temperature for 30 min. The reaction was stopped by addition of 30 μl of kinase buffer plus 25 mM EDTA and then peptide phosphorylation was measured by binding to p81 paper (Whatman) and Cerenkov counting.

To assay the activity of LCK expressed in the JCam-1.6 clones, cells were lysed using SB containing 1% Triton X-100 instead of *N*-octyl-β-D-glucopyranoside. Immunoprecipitation was carried out as for COS-18 cells. The kinase reaction was modified slightly to increase the sensitivity of the assay. The composition of the kinase buffer was: 50 mM PIPES pH 6.5, 2 mM MnCl₂, 5 mM DTT, 0.1 mg/ml BSA, and the sequence of the peptide substrate used for these assays was: H₂N-GAEEEI-YAAFFAKKK-COOH (provided by Sheldon Ratnovsky, BASF). This peptide has been defined as the optimal substrate for LCK by screening peptide libraries (Songyang and Cantley, 1995). The kinase reaction was carried out as described above and quantified by Cerenkov counting of labelled peptide bound to p81 paper.

Immunofluorescence and confocal microscopy

Transiently transfected COS-18 cells were detached with trypsin, washed with PBS and then fixed for 30 min at room temperature in 3.7% paraformaldehyde. Fixed cells were settled onto coverslips pretreated with 3-aminopropyltriethoxy silane (TESPA, Sigma), for a minimum of 2 h at 4°C. Coverslips were then incubated in 0.5% Triton X-100 in PBS for 5 min at room temperature to permeabilize the cells and then blocked for 15 min with 0.5% fish skin gelatin. After blocking, the coverslips were washed in PBS and anti-LCK antibody was added (1/100 dilution) for a 2 h incubation at room temperature, followed by an FITC-conjugated second stage antibody for 30 min. Non-specifically bound antibody was removed by extensive washing in PBS, and the coverslips were then mounted onto glass slides using a glycerol/PBS solution (Citifluor).

Confocal imaging was performed using a laser scanning head (MRC-600; Bio-Rad laboratories, Cambridge, MA) fitted to an Olympus BH-2

microscope. Fluorescein-labelled antibody was excited at 488 nm using a krypton–argon mixed gas laser (Bio-Rad) and a K1 filter. Images were collected using the Kalman filter settings of the COMOS programme (Bio-Rad). Data are presented as single optical sections.

Cell fractionation

Transiently transfected COS-18 cells, or 10×10^6 JCam-1.6 cells stably expressing LCK mutants, were resuspended in 1 ml of hypotonic buffer (10 mM Tris, 2 mM EDTA and 1 mg/ml each of chymostatin, leupeptin and pepstatin, pH 7.4), and then subjected to two successive freeze–thaw cycles. The cell suspension was homogenized on ice using a Dounce homogenizer (40 strokes) and the salt concentration was then adjusted to 150 mM NaCl. Intact cells, nuclei and other debris were pelleted by two successive centrifugations at 480 *g* for 5 min. Soluble and particulate fractions were generated following centrifugation at 100 000 *g* for 30 min. Equivalent portions of the fractionated protein were resolved by SDS–PAGE and Western blotted with anti-LCK antibody.

Sucrose gradient fractionation of cell extracts

JCam-1.6 cells (50×10^6), expressing the various LCK mutants as indicated, were washed once in PBS and then lysed for 15 min in 1 ml of MNE buffer (150 mM NaCl, 2 mM EDTA, 25 mM MES pH 6.5) containing 1% Triton X-100 and protease inhibitors, on ice. An equal volume of an 80% sucrose solution in MNE buffer was mixed with the lysates to form a 40% suspension, and a step sucrose gradient was formed by overlaying with 2 ml of 30% sucrose–MNE and 1 ml of 5% sucrose–MNE. Isopycnic equilibriation was achieved by centrifugation at 200 000 *g* for 14 h in an SW55 rotor (Beckman) at 4°C. Two 2 ml fractions were collected; one from the top of the tube that contained the 30/5% sucrose interface and the other from the bottom 2 ml, which contained the 40% sucrose fraction. Twenty five μl of each fraction was mixed with an equal volume of 2× Laemmli sample buffer and the proteins were separated by 12% SDS–PAGE, transferred onto PVDF membrane and then probed with anti-LCK antibody.

Acknowledgements

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