Tyrosine phosphorylation is one of the key covalent modifications that occur in multicellular organisms. Since its discovery more than 30 years ago, tyrosine phosphorylation has come to be understood as a fundamentally important mechanism of signal transduction and regulation in all eukaryotic cells. The tyrosine kinase Lck (lymphocyte-specific protein tyrosine kinase) plays a crucial role in the T-cell response by transducing early activation signals triggered by TCR (T-cell receptor) engagement. These signals result in the phosphorylation of immunoreceptor tyrosine-based activation motifs present within the cytosolic tails of the TCR-associated CD3 subunits that, once phosphorylated, serve as scaffolds for the assembly of a large supramolecular signalling complex responsible for T-cell activation. The existence of membrane nano- or micro-domains or rafts as specialized platforms for protein transport and cell signalling has been proposed. The present review discusses the signals that target Lck to membrane rafts and the importance of these specialized membranes in the transport of Lck to the plasma membrane, the regulation of Lck activity and the phosphorylation of the TCR.

Key words: Lck, membrane raft, T-cells, tyrosine kinase, tyrosine phosphatase.

THE DISCOVERY OF LCK

Tyrosine phosphorylation was reported as a new type of protein modification more than three decades ago during the course of research aimed at characterizing polyomavirus middle T- and v-Src-associated kinase activities (reviewed in [1,2]). Lck (lymphocyte-specific protein tyrosine kinase) was identified shortly thereafter, originally as a 56 kDa major tyrosine kinase substrate (pS6) of a Moloney murine leukaemia virus-transformed T-cell lymphoma [3,4]. The elucidation of the complete primary structure of the Lck protein revealed the presence of a conserved protein kinase domain similar to that of the tyrosine kinases v-Src and v-Yes, as well as an approximately 53% overall identity with these two kinases [5]. The pioneering discovery of Lck was destined to have a major impact on the TCR (T-cell receptor) signalling paradigm over the following years. Following the characterization of Lck as a tyrosine kinase, independent work established that Lck contributed to antigen-dependent T-cell activation by transmitting phosphotyrosine-dependent signals [6–8]. A useful strategy for studying the mechanism of TCR signalling was the generation of genetically altered Jurkat T-cell lines that were defective for this process [9]. One of the first Jurkat mutants to be characterized was the J.CaM1 clone [10], which is deficient in the induction of tyrosine phosphorylation due to a defect in the expression of functional Lck [11]. The observation that the expression of intact Lck in J.CaM1 cells restored normal TCR-mediated signal transduction indicated unambiguously that Lck is essential for this process. Consistent with the importance of Lck in T-cell regulation, some years later it was shown that Lck-knockout mice present thymic atrophy, a severe block in thymocyte development, and a dramatic reduction in the number of peripheral T-cells [12]. The fundamental role of Lck in the immune response was highlighted further by the discovery that defective expression of Lck causes severe combined immune deficiency in humans [13].

LCK STRUCTURE

The members of the Src family of non-receptor kinases have molecular masses in the range 52–62 kDa and have a similar structure (Figure 1A). They comprise six functional domains that, from the N- to the C-terminal end, include: a 9–12-amino-acid-long N-terminal SH4 (Src homology 4) domain that is myristoylated; a unique domain of 40–70 amino acids, which does not share sequence similarities with the different Src kinases; SH3 and SH2 domains, which bind other proteins; a catalytic domain referred to as the SH1 domain, with tyrosine kinase activity; and a short C-terminal regulatory region [14]. The Src family consists of eight members in humans: Src, Lck, Lyn, Yes, Fyn, Fgr, Hck and Blk [15]. Frk, which is myristoylated in rodents but not in humans, constitutes an additional Src family member in most of the mammalian species analysed (Figure 1B).

The SH4 and unique domains

Acylation of the SH4 domain attaches Src-family proteins to the cytoplasmic leaflet of the lipid bilayer. In addition...
to be myristoylated, the SH4 domain of all Src kinases, with the exception of Src and Blk, is also palmitoylated [16] (Figure 1C). Lck is myristoylated at Gly2 and palmitoylated at Cys3 and Cys5 [17]. Mutagenesis of either Gly2 or Ser6 abrogates Lck myristoylation, and, as a consequence, palmitoylation and membrane association as well [18,19].

The function of the unique domain is unclear for most members of the Src family kinases, but in Lck it mediates association with the cytoplasmic tails of the CD4 and CD8α co-receptors [20–22]. Conserved Cys-Xaa-Cys-Pro motifs in the co-receptors (where Xaa is any amino acid) and a Cys-Xaa-Xaa-Cys motif present in the Lck unique domain, but not in other Src family kinases, direct high-affinity binding. Structural analysis revealed that, whereas the co-receptor tails and the unique domain of Lck mutants respectively. Amino acid numbering corresponds to murine Lck. (B) Dendrogram showing the relatedness of the murine Src family kinases. (C) Alignment of the first ten amino acid sequence of the SH4 domain of murineSrc kinases, showing the cysteine residues (red boxes) in the vicinity of the N-terminal myristoylated glycine residue (yellow box). In the case of Hck, the aligned sequence corresponds to the p56 species. (D) Inactive and active conformations of Lck. Interactions between phosphorylated Tyr505 and the SH2 domain, and between the SH3 domain and the SH2–SH1 linker region maintain the Lck molecule in a ‘closed’ inactive state. Dephosphorylation of Tyr505 and autophosphorylation of Tyr394 convert Lck into an ‘open’ active conformation.

The SH3 and SH2 domains

In addition to binding other proteins, with specific proline-based or phosphorylated tyrosine-based sequences respectively, the SH3 and SH2 domains of Src kinases participate in intramolecular interactions that, together with phosphorylation of two specific tyrosine residues, regulate catalytic activity. One of these tyrosine residues is an autophosphorylation site located within the catalytic domain (Tyr394 in murine Lck); the other is present at the C-terminal region of the molecule (Tyr505 in murine Lck). Tyr505 or the equivalent residues in other Src kinases is crucial for regulating tyrosine kinase activity, because when it is phosphorylated it interacts with the SH2 domain and causes, in conjunction with an interaction between the SH3 domain and a Pro-Gln-Lys-Pro sequence present in the SH2–SH1 linker region, the folding of Lck in a ‘closed’ inactive conformation. In Lck, Hck, Blk and Lyn, the SH3-binding site in the SH2–SH1 linker matches the Pro-Xaa-Xaa-Pro consensus sequence generally favoured by SH3 domains, whereas in all other members of the family the second proline residue is absent, although the sequence still adopts an adequate conformation to interact with the SH3 domain [14]. The structures of Src and Hck showed that it is the SH3 domain that plays the more direct role in controlling catalytic activity [24,25]. The role of the SH2 domain appears to be secondary, with binding of the SH2 domain to the phosphorylated tail segment being important for correctly positioning the SH2–SH1 linker region to interact with the SH3 domain [26]. Similar to many protein kinases, Src family members are regulated by phosphorylation within the catalytic domain. In the case of Lck, upon dephosphorylation of Tyr505, the molecule ‘opens’ and removes the constraints upon the kinase domain, leading to autophosphorylation of Tyr394 and making the SH3 and SH2 domains available to interact with other molecules (Figure 1D).

The catalytic domain

The structure of the catalytic domain of Src kinases is similar in its essential elements to that of the active form of protein kinase A [27]. The catalytic domain of Lck (~300 residues) consists of two lobes: the smaller N-terminal lobe contains a five-stranded β-sheet and one α-helix, and the larger C-terminal lobe consists mainly of α-helices and is involved in most of the interactions with peptide substrates. ATP binds at the interface between the two lobes, with a glycine-rich strand-turn-strand segment providing a number of important contacts. Two contiguous segments of the heart of the catalytic domain are important for catalysis and regulation: the ‘catalytic segment’, which is very highly conserved and presents a number of residues involved in ATP binding and a phosphotransfer motif centred around Lys273 in Lck, and the adjacent ‘activation segment’, which contains the Lck Tyr394 residue of autophosphorylation [14].

RAFT MEMBRANES

One of the mechanisms proposed for compartmentation in biological membranes is the clustering of specific lipids, such as cholesterol and sphingolipids, into highly condensed domains or lipid rafts. As conceived originally [28], formation of these microdomains was thought to rely on the propensity of participating lipids to promote phase separation, apparently because of favourable packing interactions between saturated lipids and sterol. According to the model, these cholesterol-dependent liquid-ordered structures co-exist with liquid-disordered domains, which contain unsaturated phospholipids in cell membranes [29,30]. Membrane order can be directly assessed in cells by staining with fluorescent membrane probes, such as Laurdan, whose emission wavelength depends on the lipid microenvironment in which the molecule is immersed [31].

The tight packing of sphingolipids and cholesterol confers resistance in model membranes enriched in these lipids to detergent solubilization with non-ionic detergents [29]. Consequently, protein partitioning into lipid rafts has primarily been assessed on the basis of its association with DRM (detergent-resistant membrane) fractions [32]. However, it is of note that this association reflects solely the preference for DRMs and does not prove that the association of the proteins with the rafts precedes experimental manipulation [33]. Despite the apparent involvement of lipid rafts in a large repertoire of cellular functions [29,34,35], the fact that the use of different detergents, detergent
concentrations or fractionation protocols often leads to conflicting results, together with an active controversy about the size of the rafts, as determined by different techniques, has raised doubts about the lipid raft model [36,37]. In revisiting the raft model, protein–protein and protein–lipid interactions, in addition to the lipid–lipid interactions of the classical model, have been gaining recognition as important factors in the dynamics of raft domains. Considering the importance of the protein–lipid interactions in the raft organization, lipid rafts were renamed as membrane rafts [33,38]. Throughout the present review we distinguish between DRMs, which refer to the detergent-insoluble membrane fraction isolated from cells that are often assumed to derive from rafts, and rafts, which refer to the glycolipid-enriched condensed membrane subdomains present in the cell. Therefore association with DRMs refers to the partition of a molecule into these membrane fractions isolated by biochemical means whereas distribution in rafts refers to the presence of a molecule in membrane domains identified by microscopic analysis using lipid raft markers, such as the ganglioside GM1, or fluorescent membrane probes, such as Laurdan.

LCK ASSOCIATES WITH DRMs

Pioneering work described the isolation of DRM fractions enriched in GPI (glycosylphosphatidylinositol)-anchored proteins from T-lymphocytes [39]. These membranes were then characterized as large non-covalent complexes containing GPI-anchored proteins and Src kinases [40]. It was only after the discovery that engagement of the TCR promotes partitioning into DRMs of a series of newly tyrosine-phosphorylated substrates, including hyperphosphorylated CD3ζ, LAT (linker for activation of T-cells), phosphoinositol 3-kinase and phospholipase Cγ1, that rafts became recognized by immunologists as potential platforms for protein recruitment and cell signalling processes [41–43]. In response to appropriate antigens presented by an APC (antigen-presenting cell), T-cells polarize, forming a surface subdomain at the T-cell–APC interface known as the IS (immunological synapse) containing receptors and signalling molecules [44,45]. Despite raft lipids translocating to the IS [46], the IS contains condensed membranes [47] and TCR activation domains accumulate raft lipids [48], the proposed role of the rafts in the assembly of the signalling apparatus was challenged by single-molecule microscopic studies indicating that protein–protein interactions were sufficient for the assembly [49].

By analysing equal amounts of total protein, it was found that Lck is enriched more than 1000-fold in DRM fractions relative to the detergent-soluble fraction of Jurkat T-cells. The Lck detected in DRMs accounts for 40–60% of the total Lck in the cell [50]. The first ten amino acids of the SH4 domain of Lck, but not that of Src, which is not palmitoylated, confers targeting to DRMs (Figure 2A), indicating that the SH4 domain of Lck is sufficient for DRM incorporation [51,52]. Mutation of either Gly2 or of both Cys2 and Cys3 residues in this sequence prevents Lck from partitioning into DRMs [51,53] and inhibits the association of Lck with the GPI-anchored protein CD55/DAF (decay-accelerating factor) [54], which probably occurs in rafts since the protein moiety of both molecules resides on opposite sides of the membrane and cannot interact directly. Individual mutation of either Cys3 or Cys5 indicates that, similar to the association of Lck with membranes [18,55], Cys3 is more important than Cys5 for DRM incorporation [53,56], although palmitoylation of either cysteine residue is sufficient to ensure Lck biological activity [57]. In addition to lipid modifications, Lck-binding partners may modulate the presence of Lck in DRMs. For instance, overexpression of c-Cbl, a ligand of the Lck SH3 domain, depletes Lck from DRMs in Jurkat T-cells, whereas Lck partitioning is enhanced in c-Cbl-deficient T-cells [58].

As further evidence of the importance of Lck acylation, treatment of cells with the myristoylation inhibitor 2-hydroxymyristate was found to result in the synthesis of non-myristoylated cytosolic Lck with a reduced half-life [59]. In addition, treatment with 13-oxypalmitate, which is similar in chain length to palmitate, but has reduced hydrophobicity, does not affect Lck myristoylation, but probably substitutes for palmitic acid at the Cys3/Cys5 residues of Lck and interferes with the partitioning of Lck into DRMs [60]. The importance of palmitoylation for DRM partitioning of Lck is further supported by the observation that the addition of a six-lysine sequence to a non-palmitoylated Lck mutant was sufficient to re-establish membrane binding, but not the association with DRM-associated CD55/DAF [18].

LCK IS PRESENT IN MEMBRANE SUBDOMAINS IN T-CELLS

Membrane subdomains have been implicated in T-cell signalling, but their mechanisms of formation are not clear. Supporting the
presence of Lck in raft domains, immunofluorescence confocal microscopy indicated that Lck co-localizes in membrane patches with the ganglioside GM1, a lipid raft marker, after GM1 cross-linking [61]. These patches exhibited resistance to detergent extraction in situ, similar to DRMs. Depletion of cellular cholesterol with methyl-β-cyclodextrin did not disrupt the patches but, importantly, rendered Lck sensitive to detergent extraction, as occurs when DRMs are isolated from methyl-β-cyclodextrin-treated cells. TCR cross-linking with anti-CD3 antibodies induced a similar aggregation of Lck, as seen in GM1 patches, and Lck-mediated tyrosine phosphorylation [61]. These observations led to the proposal of a mechanism whereby TCR engagement promotes raft aggregation, which facilitates co-localization of the TCR with Lck and TCR-induced tyrosine phosphorylation.

Given the potentially large difference in radius between a raft and a single membrane protein, it is predicted that raft-associated proteins would have lower diffusion coefficients than non-raft-associated proteins. However, single-molecule dynamics analysis revealed an unexpectedly high mobility of a chimera consisting of the sequence of the first ten amino acids of Lck fused to GFP (Lck10–GFP) that incorporated efficiently into DRMs, compared with a GFP chimera of the CD2 transmembrane receptor that was excluded from DRMs [49]. Therefore the diffusional behaviours of Lck and CD2 do not correlate with their biochemical fractionation in DRMs or soluble membranes respectively. More recently, super-resolution fluorescence microscopy based on single-molecule detection analysis indicated that Lck is not randomly distributed in resting T-cells, but organized in discrete nanoclusters, the fraction of Lck in clusters being higher in activated T-cells [62]. Raft domains analysed by this technique were associated with a circular morphology. Importantly, Lck clusters were larger and less circular than were the Lck10–GFP domains in activated T-cells, and the clustering of Lck was unaltered when lipid raft accumulation was impaired [62]. In summary, these two independent observations question the presence of Lck in membrane rafts in the cell and the biological meaning of the association of Lck with DRMs.

**TARGETING OF LCK TO THE PLASMA MEMBRANE**

Lck localizes to the plasma membrane and to pericentrosomal endosomes in T-cells at steady state [63]. Lck targeting to the plasma membrane relies on the exocytic pathway and requires myristoylation and palmitoylation of its SH4 domain [64,65]. In addition to its presence at the plasma membrane, targeting of Lck to DRMs is required for T-cell signalling, since Lck mutants or Lck chimaeras unable to incorporate into DRMs were defective in T-cell signalling [62]. Raft domains analysed by this technique were associated with a circular morphology. Importantly, Lck clusters were larger and less circular than were the Lck10–GFP domains in activated T-cells, and the clustering of Lck was unaltered when lipid raft accumulation was impaired [62]. In summary, these two independent observations question the presence of Lck in membrane rafts in the cell and the biological meaning of the association of Lck with DRMs.

**REGULATION OF LCK ACTIVITY**

**Lck phosphorylation**

The ubiquitously expressed Csk (C-terminal Src kinase) is critical for phosphorylation of Lck and other Src-like kinases at the C-terminal tyrosine regulatory residue [84,85]. Csk possesses N-terminal SH3 and SH2 domains and a C-terminal kinase domain, but is devoid of the myristoylation signal, and the autophosphorylation and C-terminal sites of tyrosine phosphorylation present in the Src proteins [86,87]. Since Csk lacks any anchor for membrane association, it was unclear for
some time how cytoplasmic Csk phosphorylates raft-associated Lck and how this phosphorylation is regulated.

PAG/Cbp (phosphoprotein associated with glycolipid-enriched microdomains/Csk-binding protein) is a 68–85 kDa transmembrane adaptor protein that belongs to the TRAP (transmembrane adaptor protein) family. A very short extracellular domain and a relatively long cytoplasmic tail containing a variable number of potential tyrosine-based motifs characterize this family of proteins [88]. In addition to PAG/Cbp, the TRAP family includes LAT, LIME (Lck-interacting membrane protein) and other proteins with a similar structure and that are or are not associated with raft membranes. PAG/Cbp, which is palmitoylated, is constitutively found in DRMs. PAG/Cbp contains a tyrosine-based sequence (Tyr157) that, when phosphorylated, interacts with the SH2 domain of Csk and recruits it into DRMs. Phosphorylated PAG/Cbp, therefore, appears to function as a membrane anchor for Csk, bringing it into close proximity to raft-associated Lck to phosphorylate its Tyr505 regulatory residue [89] (Figure 3A). In resting T-cells, a high steady-state level of PAG/Cbp phosphorylation is maintained, most probably by the constitutive activity of the tyrosine kinase Fyn, which also partitions into DRMs [90]. Immediately after stimulation through the TCR, PAG/Cbp becomes rapidly dephosphorylated, resulting in the release of Csk from the membrane [89,91,92], in turn leading to Lck activation. Consistent with a role of PAG/Cbp in Lck regulation, overexpression of PAG/Cbp inhibits T-cell activation to some extent, which was not observed with PAG/Cbp mutants unable to associate with Csk [89,91]. In addition, palmitoylation-deficient PAG/Cbp, which is excluded from DRMs, does not inhibit T-cell activation when overexpressed [93]. The identity of the phosphatase(s) responsible for PAG/Cbp dephosphorylation is under debate, although CD45 [91] or SHP-2 (SH2-domain-containing tyrosine phosphatase 2) [94] are the most likely candidates.

LIME is a 31 kDa TRAP that, like PAG/Cbp, is capable of recruiting Csk to DRMs. LIME contains distinct tyrosine-based sequences (Tyr167, Tyr200, Tyr235 and Tyr254) that interact in a tyrosine phosphorylation-dependent manner with the SH2 domain of Csk (mainly Tyr167 and, to a minor extent, Tyr200), Lck (Tyr235) and Lck and Fyn (Tyr254). It seems that LIME becomes phosphorylated only after ligation of the co-receptors CD4 or CD8 [95,96]. Following CD4-mediated phosphorylation by Src kinases, LIME simultaneously associates with the SH2 domain of Lck and Fyn and, most importantly, with their negative regulator, Csk [95]. Paradoxically, LIME-associated Lck has greater enzymatic activity than the overall pool of Lck [95], probably because the phosphorylated Tyr167 of LIME-bound Lck cannot bind to the Lck SH2 domain, since the latter is already engaged by phosphorylated LIME. Consequently, the inactive conformation of Lck cannot occur in the LIME–Csk–Lck complex, and therefore, the LIME-bound Lck is maintained in an activated state, despite the presence of its inhibitor Csk in the same complex (Figure 3B). These observations imply that LIME acts as a positive regulator of Lck and, hence, of TCR-mediated signalling. Consistent with this view, overexpression of LIME in Jurkat T-cells amplifies TCR-mediated signalling [95].

The inhibitory effect of Csk on TCR signalling can be reduced by a mutation (R107K) in its SH2 domain, which is essential for the interaction of Csk with PAG/Cbp and LIME. Fusion of Csk to the myristoylation signal of Src restores the ability of the Csk mutant to inhibit TCR-triggered signals, confirming that Csk recruitment to the plasma membrane is crucial to its function [97]. As the myristoylation signal from Src does not target proteins to DRMs, targeting of Csk to rafts (as brought about by PAG/Cbp and LIME) might not be absolutely necessary for regulating the pool of Lck involved in TCR signalling. Alternatively, it is possible that the myristoylated SH4 domain of Src allows for a transient or labile association with rafts that is difficult to detect by biochemical means. In addition, despite the important role of PAG/Cbp and LIME in Lck regulation in vitro cell systems, T-lymphocytes from PAG/Cbp- and LIME-knockout mice do not show any apparent dysregulation of TCR signalling, as would be expected given the reduced levels of membrane-associated Csk [98,99]. Therefore either there are additional adaptors bringing Csk to membrane rafts [100,101] or the membrane raft-associated fraction of Csk is not essential for TCR signalling. The Csk regulatory system is even more complex since protein kinase A type 1, which is also present in DRMs from activated T-cells, phosphorylates and thereby activates DRM-associated Csk [102,103].

Lck dephosphorylation

CD45 is a heavily glycosylated transmembrane protein of 180–220 kDa that contains two tandemly duplicated protein tyrosine phosphatase homology domains in its long cytoplasmic tail [104]. The unique N-terminal region and the SH2 domain of Lck are involved in an interaction with CD45 [105]. The catalytic activity of CD45, which resides in the first phosphatase domain, is directly involved in Tyr505 dephosphorylation, counteracting the intramolecular association that down-regulates the enzymatic activity of Lck [106] (Figure 4A). Consistent with this role of CD45, the expression of a constitutively active Lck Y505F mutant rescues the block in thymic development observed in CD45-knockout mice, providing genetic evidence that the Tyr505 regulatory residue is a physiologically relevant CD45 substrate in vivo [107,108]. In addition to Tyr505, CD45 dephosphorylates the activation of the Tyr594 residue present in the catalytic domain
of Lck (Figure 4A), thereby suppressing Lck kinase activity [109,110]. The differential regulation of the two Lck tyrosine phosphorylation sites by CD45 is critical for T-cell receptor signalling responses [111].

There are conflicting reports about the association of CD45 with DRMs [112,113]. One study found that CD45 is excluded from DRMs isolated using 1% Triton X-100 and that Lck is identically phosphorylated in DRMs and soluble membranes in cells lacking CD45. These findings were interpreted as meaning that the hyperphosphorylation of Tyr505 and the lower kinase activity of DRM-associated Lck are due to the exclusion of CD45 from rafts [112]. A different study reported that approximately 5% of CD45 is detected in DRMs isolated using 0.5% Triton X-100. As the percentage of CD45 in DRMs was found to diminish upon TCR stimulation, it was proposed that exclusion of CD45 from rafts might limit the levels of activated Lck and thus possibly the duration of T-cell responses [113]. Using CD45 chimaeras bearing ectodomains of different sizes, a small fraction of wild-type CD45 or of CD45 proteins bearing large ectodomains (such as that of CD43) was observed to partition into DRMs and produce higher levels of pTyr394 Lck and efficient TCR-mediated signalling than CD45 chimaeras with small ectodomains (such as that of Thy-1 or CD2) [114]. Therefore the ectodomain of CD45 appears to be important for determining CD45 partitioning into DRMs and CD45-mediated dephosphorylation and activation of Lck. The kinetic-segregation model [115,116] proposes that random interactions of Lck with itself, with CD45 and with Csk generate differentially phosphorylated forms of Lck in resting T-cells. In turn, random interactions of the TCR with Lck and CD45 produce constitutive phosphorylation and dephosphorylation processes respectively, that lead to basal low levels of tyrosine-phosphorylated TCR. According to this model, the interaction of a T-cell with an APC in the presence of the appropriate antigen produces the formation of close cell–cell contact zones driven by interaction of small surface molecules that alter the local balance between these constitutive processes by inducing size-dependent segregation of the TCR from proteins with large ectodomains, such as CD45. This segregation would result in an extended half-life of phosphorylated TCR leading to cell signalling.

The role of membrane rafts in the dephosphorylation of Lck by CD45 has also been addressed by using chimaeras of the cytoplasmic tail of CD45 containing the two tyrosine phosphatase homology domains fused to the SH4 domain of either Lck or Src, which target the chimaeras to DRMs or to detergent-soluble membranes respectively [117]. Lck dephosphorylation occurred to a similar extent regardless of the presence of the CD45 chimaera in DRMs. In addition, no differences were found in Lck activity in cells expressing either chimaera, probably due to compensatory effects that regulate Lck dephosphorylation positively at Tyr505 and negatively at Tyr506. The results of that study led to the conclusion that CD45 partitioning into rafts does not play a major role in regulating baseline dephosphorylation and Lck activity [117]. In T-cells from patients with SLE (systemic lupus erythematosus), there is a more pronounced inclusion of CD45 in DRMs, and CD45 is maintained in these membranes even after the TCR is stimulated. Consistent with the increased presence of CD45 in DRMs [118], active Lck is increased, despite the reduced content of total Lck in these cells [119–121]. The increased amounts of active Lck in DRMs correlated with high levels of hyper-responsive T-cells, which is consistent with the low activation threshold previously observed in SLE T-cells [122]. Importantly, treatment with atorvastatin, an inhibitor of cholesterol biosynthesis, disrupted the co-localization of Lck and CD45 in GM1-enriched domains, reduced the levels of active Lck, and reversed many of the signalling defects observed in SLE T-cells [123]. It was therefore proposed that changes in the partitioning of CD45 into raft-enriched membranes are critical for Lck tyrosine kinase activity and T-cell responsiveness at least in SLE cells [124].

Two non-transmembrane phosphatases, PEP (PEST domain-enriched tyrosine phosphatase), which is the mouse homologue of human PTPN22, and SHP-1 also act at the activating Tyr505 residue of Lck [125–127]. In addition to Lck, PEP and SHP-1 dephosphorylate other substrates relevant in T-cell signalling, including ZAP-70, Vav, Grb2, c-Cbl and SLP-76 (SH2 domain-containing leucocyte protein of 76 kDa) [128,129]. PEP consists of an N-terminal catalytic domain and a long C-terminal region containing proline-rich motifs. PEP forms a complex with Csk through the first proline-rich motif of PEP and the SH3 domain of Csk [130]. In this way, PEP can work in concert with Csk to convert active pTyr505 Lck into its inactive pTyr506 Lck form (Figure 4B) [131]. Consistent with the role of PEP in Lck regulation, PEP-knockout mice demonstrated enhanced activation of Lck and increased expansion of the effector/memory T-cell pool [132]. SHP-1 consists of two tandem SH2 domains at the N-terminal part followed by the catalytic domain and a C-terminal tail [133]. In resting cells, the N-terminal SH2 domain sterically inhibits the activity of SHP-1 by interacting with the catalytic domain. In T-cell lines and primary T-cells, 20–30% of SHP-1 partitions into DRMs [134,135]. The fusion of the SH4 domain of Lck to the N-terminus of SHP-1 targets the molecule to DRMs and results in profound defects in TCR-induced tyrosine phosphorylation and cell activation [130]. Similarly, when SHP-1 was targeted to DRMs via a LAT-anchoring sequence, LAT phosphorylation and other downstream events in the TCR signalling cascade were strongly inhibited, although the proximal events, including the phosphorylation of CD3 and ZAP-70, were unaffected [126]. These two examples of ‘forced’ targeting of SHP-1 to DRMs could be interpreted simply as meaning that SHP-1 has to be in close proximity to its substrates or as that the association of SHP-1 to specific rafts is important for its biological activity. A feedback loop regulation of Lck by SHP-1 initiated by TCR signals was proposed [136].
Supporting this proposal, it was found that weak or antagonistic TCR ligation results in a negative feedback response consisting of rapid Lck-mediated phosphorylation of SHP-1 at Tyr$^{564}$, binding of phosphorylated SHP-1 to the SH2 domain of Lck and Lck inactivation by SHP-1-mediated dephosphorylation of Tyr$^{306}$ of Lck (Figure 4C). Conversely, strong or agonistic TCR ligation triggers a positive feedback loop by which the mitogen-activated kinase ERK (extracellular-signal-regulated kinase) is rapidly activated and phosphorylates Lck on Ser$^{59}$, and this modification prevents SHP-1 binding to Lck, thus keeping Lck active to sustain TCR signals [137]. The characteristics of these pathways suggest that they constitute an important part of the mechanism allowing T-cells to discriminate between self and foreign ligands.

**LCK, RAFTS AND TCR SIGNALLING**

In unstimulated cells, Lck may exist in four different forms according to the phosphorylation at the Tyr$^{306}$ activation and Tyr$^{505}$ inhibitory sites: ‘closed-inactive’ (Tyr$^{306}$/Tyr$^{505}$), with the SH2–pTyr$^{505}$ intramolecular interaction locking the catalytic site into an inactive state; ‘primed’ (Tyr$^{306}$/Tyr$^{505}$), with the interaction between the SH3 domain and the Pro–Xaa–Xaa–Pro in the SH2–SH1 linker region blocking the catalytic site; ‘mono-phosphorylated active’ (pTyr$^{306}$/Tyr$^{505}$), with Lck in an optimal conformation for catalysis; and ‘double-phosphorylated active’ (pTyr$^{306}$/pTyr$^{505}$) with an open conformation and in vitro kinase activity similar to that of pTyr$^{306}$/Tyr$^{505}$ [138]. In primary T-cells, the ‘primed’ Tyr$^{306}$/Tyr$^{505}$ form accounts for approximately 48% of total Lck, whereas the active pTyr$^{306}$/Tyr$^{505}$ and pTyr$^{306}$/pTyr$^{505}$ forms represent approximately 17% and 21% respectively of the total Lck content [138].

It has been determined that approximately 60% and 70% of total pTyr$^{306}$ and pTyr$^{505}$ Lck respectively are associated with DRMs from primary human T-lymphocytes [139]. Although CD4 associates with both phosphorylated Lck forms, it preferentially interacts with pTyr$^{306}$ Lck. It was proposed that these CD4/Lck complexes might serve as primer initiation complexes allowing T-lymphocytes to respond promptly [139]. On the other hand, LAT, which is constitutively present in DRMs, appears to interact preferentially with the active Tyr$^{505}$-dephosphorylated form of Lck [140], probably to recruit it to the rafts and regulate its inactivation by phosphorylation at this residue by PAG/Cbp-associated Csk. There are conflicting reports about the relative levels of pTyr$^{306}$ and pTyr$^{505}$ after TCR engagement. Although one study found that TCR engagement does not modify these levels [138], other researchers described a transient increase in the levels of pTyr$^{306}$ and a reduction in those of pTyr$^{505}$ [139]. In any case, the relatively high amounts of active pTyr$^{306}$ Lck constitutively present in unstimulated cells seem sufficient to initiate TCR signalling [138,141].

A central question is how, although it can do so within seconds of TCR engagement, activated Lck is prevented from extensively phosphorylating the ITAMs (immunoreceptor tyrosine-based activation motifs) present within the cytosolic tails of the CD3 subunits of the TCR in unstimulated cells [142]. Although tyrosine phosphorylation occurs very early after TCR engagement, it is not the first event because it is preceded by a conformational change in the TCR/CD3 complex that occurs independently of tyrosine phosphorylation. This change involves the exposure of hidden sequences within a subunit that allows binding of Nck to a proline-rich motif present in the cytoplasmic tail of the CD3ξ [143]. Therefore it is conceivable that the TCR conformational change might also control the accessibility of the ITAMs to Lck. Using FRET and a Lck biosensor, it has been recently found that, although the total levels of pTyr$^{306}$ Lck are unaltered, there is a local activation through conformational change of 20% of the total Lck molecules at sites of TCR engagement [144]. Super-resolution fluorescence microscopic studies based on single molecule detection determined that Lck is not randomly distributed in resting cells, but is organized into discrete nanoclusters [62]. The size of the clusters, but not their number, changes upon TCR engagement, the proportion of Lck in clusters being higher in activated than in resting cells. Conformation-driven Lck clustering is highly dynamic, so that TCR triggering results in Lck clusters that contain phosphorylated TCR, but exclude the phosphatase CD45. Importantly, Lck clustering is regulated via its conformational states with the open and closed conformations respectively inducing and opposing clustering [62]. In contrast, association with raft domains and protein–protein networks were found to be not sufficient or necessary for Lck clustering. These findings led to the proposal that Lck conformational states regulate local Lck clustering and distribution to enable frequent TCR–Lck interactions and TCR phosphorylation [62].

The observations that the assembly of the signalling machinery ([49] or the local clustering of Lck [62] do not require association with raft domains do not rule out the possibility that raft membranes are the milieu where these processes occur, in keeping with the existence of condensed domains and raft lipids at TCR activation domains [46–48]. In this sense, Lck has been detected in two distinct DRM fractions obtained upon extraction with Brij58 that were named ‘heavy DRMs’ and ‘light DRMs’, the latter being equivalent to standard DRMs [141]. In unstimulated cells, the activated pool of pTyr$^{306}$ Lck localized to heavy DRMs. After TCR/CD4 aggregation, a fraction of active pTyr$^{306}$ Lck and CD3ξ, and presumably the rest of the TCR components, originally associated with ‘heavy DRMs’ redistributed to ‘light DRMs’, which were depleted in CD45 to allow high levels of ITAM phosphorylation [141]. These results, therefore, argue for a role of distinct raft domains in TCR signalling [145]. The actin cytoskeleton might regulate the maintenance of the distinct raft domains and their rearrangement after TCR triggering [52,146,147].

In summary, ectodomain-size exclusion in close contact zones and changes in: (i) the accessibility of the ITAMs; (ii) the local clustering and catalytic activity of Lck; and (iii) the compartmentation of the TCR, Lck and CD45 into different membrane domains, probably integrate to prevent inopportune signalling in resting T-cells and to enable efficient and rapid ITAM phosphorylation by Lck after TCR ligation (Figure 5).

**CLOSING REMARKS**

Thirty years have passed since the discovery of the tyrosine kinase Lck. The first two decades of Lck research yielded a plethora of information about its structure, regulation and function. In the last decade, considerable efforts have been made to study the membrane microenvironment in which a large fraction of Lck localizes. This specialized milieu probably made of raft lipids plays an important role in transporting Lck to the plasma membrane, in regulating the cycles of Lck phosphorylation/dephosphorylation, and in compartmentalizing the activity of Lck to specific sites of the cell. Despite the current controversy about whether membrane rafts even exist in intact cells, it is indisputable that these studies have boosted Lck research and have contributed to our appreciation of the importance of membranes in developing our models of Lck regulation and function. Now might be the time to characterize the protein...
machinery, other than MAL, Unc119 and Rab11, that controls Lck transport to the plasma membrane, to use lipidomics to identify the lipids that form the building blocks of the rafts involved in Lck regulation and TCR signalling, and to investigate how these domains remodel after TCR triggering. The achievement of the latter objective will shed light on the role of lipids in these processes and may help to explain how clustering and conformational changes of Lck and TCR occur to regulate TCR phosphorylation and signalling.

**FUNDING**

Research in M.A.A’s laboratory is supported by the Ministerio de Economía y Competitividad, Spain [grant numbers BFU2012-32532 and CONSOLIDER COAT CSD2009-00016].

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The Authors Journal compilation © 2013 Biochemical Society

Received 1 April 2013/31 May 2013; accepted 24 June 2013
Published on the Internet 9 August 2013, doi:10.1042/BJ20130468