REVIEW ARTICLE

Positive and negative regulation of T-cell activation through kinases and phosphatases

Tomas MUSTELIN* and Kjetil TASKÉN†[1]

*Program of Signal Transduction, Cancer Center, The Burnham Institute, 10901 North Torrey Pines Road, La Jolla, CA 92037, U.S.A., and †Department of Medical Biochemistry, Institute of Basic Medical Sciences, University of Oslo, P.O.B. 1112, Blindern, N-0317 Oslo, Norway

The sequence of events in T-cell antigen receptor (TCR) signalling leading to T-cell activation involves regulation of a number of protein tyrosine kinases (PTKs) and the phosphorylation status of many of their substrates. Proximal signalling pathways involve PTKs of the Src, Syk, Csk and Tec families, adapter proteins and effector enzymes in a highly organized tyrosine-phosphorylation cascade. In intact cells, tyrosine phosphorylation is rapidly reversible and generally of a very low stoichiometry even under induced conditions due to the fact that the enzymes removing phosphate from tyrosine-phosphorylated substrates, the protein tyrosine phosphatases (PTPases), have a capacity that is several orders of magnitude higher than that of the PTKs. It follows that a relatively minor change in the PTK/PTPase balance can have a major impact on net tyrosine phosphorylation and thereby on activation and proliferation of T-cells. This review focuses on the involvement of PTKs and PTPases in positive and negative regulation of T-cell activation, the emerging theme of reciprocal regulation of each type of enzyme by the other, as well as regulation of phosphotyrosine turnover by Ser/Thr phosphorylation and regulation of localization of signal components.

Key words: Csk, kinase, Lck, phosphatase, T-cell, Zap.

INTRODUCTION

T-lymphocytes play a central role in the immune response, both as direct effector cells and as regulatory cells that modulate the functions of numerous other cell types, primarily those that participate in the body's defence mechanisms. This regulatory function is provided either through direct cell–cell contact or via the secretion of various cytokines. Thus the proper function of T-cells is essential for the maintenance of normal homoeostasis within and outside the immune system. Conversely, abnormalities in their function can lead to immunological diseases, e.g. autoimmunity, allergies or immunodeficiencies. The primary event leading to the activation and differentiation of mature T-cells is the triggering of their antigen-specific T-cell receptor (TCR) by its specific ligand, which consists of a processed antigenic peptide presented in association with MHC molecules on the surface of antigen-presenting cells or appropriate target cells. This event triggers several signal transduction pathways that involve second messengers, protein kinases, protein phosphatases and other enzymes and key intermediates [1–4]. This signalling cascade culminates with the induction of gene transcription according to defined genetic programmes that are characteristic of the different T-cell subsets, leading to the differentiation and proliferation of these cells. In addition, the proper development and selection of immature T-cells in the thymus also depends on similar signalling events that determine the selection of T-cells with the appropriate antigen specificity.

OVERVIEW OF T-CELL ACTIVATION AND THE PROTEIN TYROSINE KINASES (PTKs) IMPLICATED

The key recognition and activation element during physiological T-cell responses to antigen is a complex receptor, consisting of two clonally distributed, highly polymorphic heterodimeric $\alpha\beta$ or $\gamma\delta$ subunits of the TCR, the three invariant CD3 polypeptides, and two additional homodimeric (ζ-ζ) or heterodimeric (ζ-η) subunits. The function of the $\alpha\beta$ (or $\gamma\delta$) TCR is to recognize and specifically bind antigenic peptides presented by MHC molecules, while the CD3 complex, ζ and η transduce the signal generated by ligand binding to the TCR. The various chains associate non-covalently to form the complete and functional receptor.

The identification of a number of PTKs and many of their substrates, and understanding of the sequence of events leading to activation of downstream signalling pathways, represents a major advance in our understanding of TCR signalling. In the last 10 years, it has also become clear that many other receptors of the immunoglobulin superfamily employ a similar mechanism of signal transduction, which involves PTKs of the Src, Syk, Csk and Tec families, adapter proteins and effector enzymes in a highly organized tyrosine-phosphorylation cascade (Figure 1). It

Abbreviations used: Cbp, Csk-binding protein; ERK, extracellular signal-regulated protein kinase; HePTP, haematopoietic PTPase; ITAM, immunoreceptor tyrosine-based activation motif; ITIM, immunoreceptor tyrosine-based inhibitory motif; JNK, c-Jun N-terminal kinase; KIM, kinase-interaction motif; LMPTP, low-M_r PTPase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; MKP, MAP kinase phosphatase; PAG, phosphoprotein associated with glycosphingolipid-enriched membrane domains; PEP, PEST-enriched protein tyrosine phosphatase; PKA, protein kinase A; PTK, protein tyrosine kinase; PTPase, protein tyrosine phosphatase; PTP-MEG2, PTPase from megacaryocytes 2; PTP-PEST, PTPase with PEST sequences; SH2, Src homology 2; SH3, Src homology 3; SHP1, SH2-domain-containing protein tyrosine phosphatase 1; SLP-76, SH2-domain-containing leucocyte protein of 76 kDa; SMAC, supramolecular activation cluster; TCPTP, T-cell PTPase; TCR, T-cell receptor; VHR, VH1-related; VHX, VHR-related MKPX.

[1] To whom correspondence should be addressed, at: Department of Medical Biochemistry, University of Oslo, P.O.B. 1112, Blindern, N-0317 Oslo, Norway (e-mail kjetil.tasken@basalmed.uio.no).

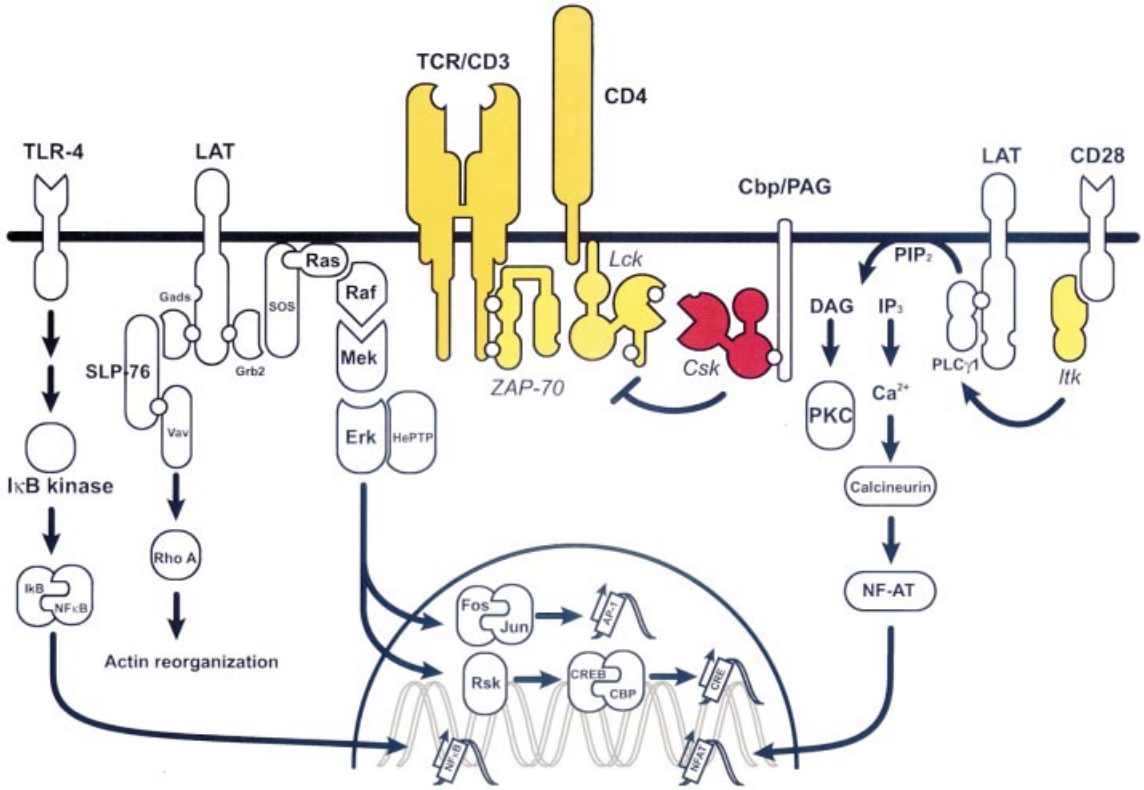

Figure 1 Intracellular signalling pathways and kinases involved in T-cell activation

Triggering of the TCR and co-ligation of the CD4 (or CD8 on cytotoxic T-cells) releases Lck from inhibition by Csk (displaced, red), leading to phosphorylation of the TCR and recruitment and activation of ZAP-70, which phosphorylates LAT (linker for activation of T-cells). LAT then ligates phospholipase Cγ1 (PLCγ1), Grb2/Sos/Ras and Gads/SLP-76 (SH2-domain-containing leucocyte protein of 76 kDa)/VAV and activates the respective downstream signalling pathways of Ca^{2+}/InsP_3 (IP_3), Ras/Raf/ERK and RhoA, leading to gene regulation, proliferation and actin-reorganization responses. This involves activation of the important tyrosine kinases of the Src family kinase (SFK) (Lck) and Syk (ZAP-70) families as well as the release from inhibition by the tyrosine kinase Csk. Co-activation via B7 interaction with CD28 involves the Tec family of PTKs (Itk), whereas activation via the Toll receptor pathway (TLR-4) or cellular stress triggers the nuclear factor κB (NFκB) pathway involving a Ser/Thr kinase (IκB kinase). PTKs are indicated in italics with yellow or red backgrounds to indicate activating or inhibiting effects, respectively. NFAT, nuclear factor of activated T-cells; PIP_2, PtdInsP_2; PKC, protein kinase C; CREB, cAMP-response-element-binding protein; CBP, CREB-binding protein; DAG, diacylglycerol.

is important to note, however, that this cascade is also equally dependent on the class of enzymes that remove phosphate from the PTK substrates (including from the PTKs themselves), the protein tyrosine phosphatases (PTPases). In intact cells, tyrosine phosphorylation is rapidly reversible and generally of a very low stoichiometry even under induced conditions. It follows that a relatively minor change in the PTK/PTPase balance can have a major impact on net tyrosine phosphorylation and thereby on activation and proliferation of T-cells. Our understanding of the PTPases clearly lags behind the current state-of-the-art insights into the PTKs. This review focuses on PTKs and PTPases, the emerging theme of reciprocal regulation of each type of enzyme by the other, as well as regulation of phosphotyrosine turnover by Ser/Thr phosphorylation.

Initiation of the TCR signalling cascade

It is generally agreed that a key initiating event in T-cell activation by antigen is the increased phosphorylation of immunoreceptor tyrosine-based activation motif (ITAM) tyrosines in TCR subunits by the Src family kinases Lck and Fyn (and perhaps c-Yes). Exactly how the TCR couples antigen recognition to this phosphorylation is not clear. Proposed models include (i) juxtaposition of Lck to the TCR mediated by CD4 or CD8 [5–11], (ii) increased local concentrations of kinases and their substrates following receptor oligomerization and lipid raft coalescence [12–17] and (iii) the active exclusion of Csk and PTPases (discussed below). These mechanisms are not mutually exclusive, and probably all operate in concert.

In vitro, both Lck and Fyn can phosphorylate ITAM tyrosines of the ζ chain, but they do not phosphorylate all residues equally well (T. Mustelin, unpublished work): Fyn seems to phosphorylate only one site with high affinity, while Lck readily phosphorylates four or five other sites. In Lck-deficient cell lines, such as the Jurkat-derived JCaM1, TCR triggering results in a minimal (but detectable) ζ-chain phosphorylation, some ZAP-70 recruitment, but no ZAP-70 phosphorylation [18–21]. A normal ζ phosphorylation and ZAP-70 response is restored upon re-expression of Lck [19]. Thus it seems that Lck is the kinase responsible for much, if not all, of the ITAM phosphorylation that follows TCR triggering in T-cells. Fyn may catalyse some ITAM phosphorylation in the absence of Lck, but is largely unable to compensate for the loss of Lck. It has been reported that Syk also can phosphorylate ITAMs [22], but this is probably of relatively

minor importance in most T-cell populations, which have very low or undetectable levels of this PTK.

ITAM phosphates undergo a rapid turnover in intact T-cells. Thus increased ITAM phosphorylation following TCR triggering could be achieved by elevated rates of phosphorylation (as is generally believed), reduced dephosphorylation or a combination of the two. There is evidence that CD45 may be involved in ζ-chain dephosphorylation [23]. It has also been proposed that Src homology 2 (SH2)-domain-containing protein tyrosine phosphatase 1 (SHP1) catalyses this reaction [24,25], but the evidence for a direct effect is lacking. It is likely that the main responsibility for ζ dephosphorylation is carried by another as-yet-unidentified PTPase.

Recruitment and activation of ZAP-70

It seems that the main purpose of ITAM phosphorylation is to create docking sites for the tandem SH2 domains of the ZAP-70 kinase, and in some T-cells also for the related Syk kinase. Indeed, the ITAMs are necessary and sufficient to transduce seemingly complete activation signals by themselves when introduced into chimaeric transmembrane proteins that can be oligomerized by extracellular stimuli, e.g. monoclonal antibodies [26,27]. However, the need for ITAM phosphorylation can be bypassed by chimaeric receptors bearing ZAP-70 (or Syk) as their intracellular component [28]. Co-cross-linking of the ZAP-70 chimaera with a chimaera having a Src family kinase caused T-cell activation and cytotoxicity (but no ITAM phosphorylation). In contrast, cross-linking of Lck or Fyn chimaerae alone had little effect [28].

Doubly phosphorylated ITAMs bind ZAP-70 (or Syk) with such a high affinity that other SH2-domain-containing signalling molecules are excluded [29,30]. However, a number of other signalling molecules with SH2 domains, such as Grb2, Ras GTPase-activating protein, phosphoinositide 3-kinase, Shc and Src-family PTKs, have been shown to bind singly phosphorylated ITAMs [31–34]. Thus, during sequential phosphorylation of ITAM tyrosines in the TCR complex, it is possible that other molecules bind transiently to monophosphorylated ITAMs at early steps, only to be replaced by ZAP-70 upon addition of a second phosphate within each ITAM. In this context, it should be pointed out that the stoichiometry of individual ITAM phosphorylation under physiological conditions is unknown and that alterations in this stoichiometry correlate with the biological outcome of TCR triggering [35–37].

Once recruited, ZAP-70 is activated by phosphorylation, at Tyr-493 in its activation loop, by Lck [38,39]. Due to the presence of 10 ITAMs in the TCR complex, up to 10 ZAP-70 molecules may cluster on the fully phosphorylated receptor. Upon phosphorylation at Tyr-493 by Lck, these bound ZAP-70 molecules autophosphorylate, presumably *in trans*, to create docking sites for SH2-domain-containing signalling proteins [40–43].

The Src-family PTKs are also responsible for recruitment and activation [44,45] of another class of cytoplasmic PTKs, the Tec-related kinases Itk/Emt and Txk/Rlk, which are directly involved in phosphorylation and activation of phospholipase Cγ1 (Figure 1) [46,47]. It also appears that Src-family PTKs have many other substrates, including cytoskeletal proteins, adapters and other signalling molecules.

Importance of ZAP-70 for T-cell function

A very important advance in our understanding of the importance of Syk-family PTKs in leucocyte signalling and development was the discovery of patients lacking a functional *ZAP-70* gene [48–50], as well as the generation of *zap-70*-null allele [51] and *syk*-null allele [52,53] mutant mice. Lack of ZAP-70 in humans leads to a severe immunodeficiency characterized by the absence of $CD8^+$ T-cells and TCR-unresponsive mature $CD4^+$ T-cells. Mice lacking ZAP-70 are also deficient in the production of $CD4^+$ T-cells, while the natural killer cells are unaffected. Mice lacking Syk, on the other hand, died *in utero* from massive haemorrhage, while $RAG2^{-/-}$ mice reconstituted with fetal liver cells from the $syk^{-/-}$ mice failed to develop B-cells [52,53] and some intra-epithelial TCR$\gamma\delta^+$ T-cells [54]. Most other T-cells (as well as other leucocyte types) appear normal, although detailed signalling studies have not yet been carried out with them. Significantly, double knock-out mice [55] show a much more severe and early arrest in thymic development of T-cells. Thus it appears that Syk can compensate for ZAP-70 during thymocyte development, a notion that is supported by the higher level of Syk expression in thymocytes compared with mature T-cells [56]. These largely non-overlapping requirements for Syk and ZAP-70 clearly reflect differences in expression pattern and differences in biological function. This question is particularly pertinent in cells expressing more equal levels of both PTKs, such as TCR$\gamma\delta^+$ T-cells and natural killer cells.

Physiological substrates of ZAP-70

Despite the relatively high degree of amino acid identity between their kinase domains, ZAP-70 and Syk differ substantially in their substrate specificity [57–59]. It appears that ZAP-70 has a much more narrow substrate spectrum. Indeed, ZAP-70 seems to have only one well-established substrate, the adapter protein LAT (linker for activation of T-cells; Figure 1) [60–62]. Another adapter protein, SLP-76 (SH2-domain-containing leucocyte protein of 76 kDa) [63], is often mentioned as a ZAP-70 substrate, but to the best of our knowledge there is no published evidence for this. Instead, there are reports that SLP-76 is phosphorylated by Tec-family PTKs [64–67]. It also seems that many substrates are phosphorylated by Src- or Tec-family PTKs, rather than by ZAP-70.

Downstream signalling pathways

Numerous tyrosine-phosphorylation events follow the triggering of the TCR and the mobilization of the PTKs discussed above. Some of these phosphorylation reactions are unique to T-cells or lymphoid cells, whereas others are more or less generic to the activation of any mammalian cell. The increased phosphorylation of PTK substrates leads to the activation of many signalling pathways, morphological and functional changes, cell-cycle progression and the activation of numerous genes (Figure 1). A major challenge in T-cell biology is to define the exact contribution of each signalling protein to the pleiotropic T-cell activation response, as well as to understand the spatiotemporal interplay between signalling molecules and pathways.

REGULATION OF Src-FAMILY PTKs

Since Lck and Fyn, as well as other Src-family PTKs in other cell types, play such crucial role at the very beginning of receptor-mediated signal transduction it is not surprising that they are very tightly regulated at all possible levels (reviewed in [6]) Here we will only discuss the regulation by reversible tyrosine phosphorylation.

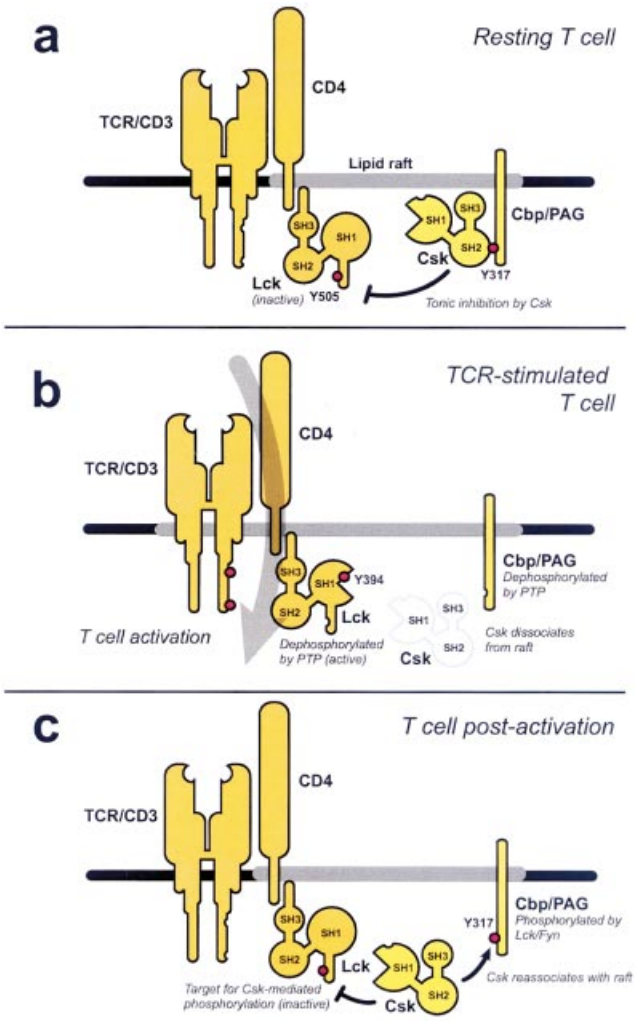

Figure 2 Csk is temporarily 'sent off duty' during T-cell activation

In resting normal T-cells (**a**), Csk is present in lipid rafts through interaction with phospho-Tyr-317 in Cbp/PAG. This imposes a tonic inhibition of T-cell activation through Csk-mediated phosphorylation of the Lck regulatory site (Tyr-505). Engagement of the TCR (**b**) results in dephosphorylation of Cbp by an unknown PTPase, dissociation of Csk from lipid rafts and displacement from its substrate Lck, leading to activation of Lck and allowing for initiation of the TCR-induced tyrosine-phosphorylation cascade. However, after 2–5 min of activation (**c**), Cbp/PAG Tyr-317 is re-phosphorylated by Lck and/or Fyn thereby recruiting Csk back into lipid rafts. Together with Tyr-394 dephosphorylation by PEP or SHP-1, this terminates Lck and Fyn activity and turns off TCR signalling.

Positive regulation of Lck and Fyn through reversible tyrosine phosphorylation

An important mode of regulation of Src-family PTKs is by phosphorylation at a conserved tyrosine residue within the catalytic domain, Tyr-394 in Lck and Tyr-417 in Fyn(T). It is generally believed that Src-family PTKs autophosphorylate at this site, presumably *in trans*, but there is also evidence that another PTK may be involved [68]. Phosphorylation at this site in Lck relieves an intramolecular block of substrate binding and also provides a conformational change in the so-called 'activation loop' (where Tyr-394 resides) to position the catalytic amino acid residues for optimal catalysis [69]. Although the stoichiometry of Tyr-394 phosphorylation in intact T-cells is very low [70,71], this event is crucial for substrate phosphorylation by Lck and for transformation by oncogenic Lck [72,73]. The low stoichiometry is mainly due to rapid dephosphorylation [74].

Suppression of Src-like PTKs by Csk

Both Lck and Fyn (as well as other Src-family PTKs) are negatively regulated by phosphorylation at a conserved C-terminal regulatory tyrosine residue; Tyr-505 in Lck and Tyr-528 in Fyn(T) ([75–77], reviewed in [6]). When phosphorylated, the tail tyrosine binds to the SH2 domain of the same kinase molecule thereby forcing it into an inactive conformation [78]. The crystallization of C-terminally phosphorylated Src [79] and Hck [80] also revealed that the Src homology 3 (SH3) domain is important for stabilizing the kinase domain in the inactive conformation by binding to a region in the linker between the SH2 domain and the kinase domain. At least in T-cell lines, approximately half of all Lck molecules are phosphorylated at Tyr-505 [81], but changes in this phosphorylation have not been observed during T-cell activation. Instead, the phosphotyrosine content of Lck increases after T-cell activation [82]. This suggests that the role of Lck in TCR signalling does not involve acute dephosphorylation at Tyr-505.

The PTK responsible for the suppressive phosphorylation of Lck at Tyr-505 and Fyn at Tyr-528 is the Csk kinase, a widely expressed 50 kDa enzyme [83–86]. At present, Csk is the only PTK known to have this specificity. The finding that disruption of the *csk* gene is lethal [87,88] and that cells recovered from the early embryos contain greatly activated Src-family PTKs, suggests that no other gene can fully compensate for the loss of *csk* during embryogenesis. Nevertheless, it is possible that other Csk family members participate in the suppression of Src-family PTKs in certain tissues or cell types.

Consistent with a negative role of Csk in the regulation of Src-family PTKs, Chow and collaborators [89] showed that overexpression of Csk in T-cells caused a marked reduction of TCR-induced tyrosine phosphorylation and interleukin-2 production. Csk required both its SH2 and SH3 domains for this activity, which was enhanced by targeting Csk to the plasma membrane [89]. One explanation for the requirement of the SH3 domain comes from the observation [90] that Csk specifically associates through its SH3 domain with the PEST-enriched protein tyrosine phosphatase (PEP). Thus the targeting of Csk to the plasma membrane may not only increase its access to Src-related PTKs but also increase the dephosphorylation of targets for PEP (e.g. Lck or Fyn) in the membrane.

Spatiotemporal regulation of Csk localization releases Lck from inhibition and allows T-cell activation to occur

Although Csk has a similar organization to that of Src-family PTKs, with SH3, SH2 and kinase domains, Csk differs from the Src kinases in that it lacks the C-terminal regulatory tyrosine and autophosphorylation sites as well as the N-terminal lipid-modification sequence that targets members of the Src kinase family to membranes. For this reason it has for some time been unclear how this ubiquitously expressed cytosolic kinase effectively regulates the membrane-associated Src kinases Lck and Fyn even though Csk has been shown to target to sites of Src activity in fibroblasts [91]. The recent cloning and characterization of a transmembrane adapter protein, Csk-binding protein (Cbp) or phosphoprotein associated with glycosphingolipid-enriched membrane domains (PAG), which is associated with lipid rafts and has an ability to bind the SH2 domain of Csk, has provided new insight into the Csk-mediated regulation of Src kinases [92,93].

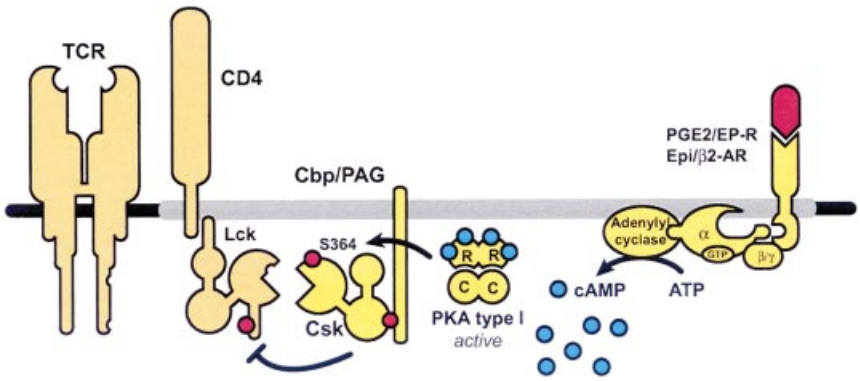

Figure 3 A G-protein-coupled receptor/cAMP/PKA/Csk pathway negatively regulates Lck signalling in lipid rafts

In resting T-cells Csk is targeted to lipid rafts through engagement of its SH2 domain to Cbp/PAG. This creates a constitutive activation of Csk and inhibition of Lck and Fyn. The activation status of raft-associated PKA in T-cells is not known, but it is likely that small and local increases in cAMP can produce small peaks in activity and thereby modulate the phosphorylation status of Ser-364 (S364) of Csk and activate Csk. Furthermore, stimulation of the receptor–G-protein–adenylate cyclase complex leads to a solid activation of PKA, resulting in a highly active form of Csk that represses Lck-dependent signalling through the TCR. PGE2, prostaglandin E_2; EP-R, PGE_2 receptor; Epi, epinephrine; β2-AR, β_2-adrenergic receptor.

Cbp/PAG is ubiquitously expressed and was originally observed as an 80 kDa phosphoprotein present in lipid rafts [94–96], and links Cbp and rafts via EBP50 to the actin cytoskeleton [97,98]. Furthermore, Cbp/PAG has a palmitoylation site and a cytoplasmic tail with 10 sites for tyrosine phosphorylation that can be phosphorylated by Lck and Fyn in T-cells. However, as yet, only Csk, Fyn and ezrin/radixin/moesin-binding phosphoprotein 50 (EBP50) have been reproducibly found to interact with Cbp/PAG *in vivo* and Tyr-317 (Tyr-314 in mouse and rat) is identified as the binding site for the SH2-domain of Csk, while Fyn seems to interact independently of the Cbp/PAG phosphotyrosine status. Interestingly, stimulation of T-cells with antibodies towards CD3 alone or in combination with CD28 induces dephosphorylation of Cbp/PAG resulting in decreased Csk content in lipid rafts [92,99]. We have further demonstrated that Csk association with Cbp/PAG is transient and correlates with the kinetics of T-cell activation [99]. Overexpression of kinase-deficient Csk, which displaces endogenous Csk from lipid rafts, has stimulatory effects on ζ-chain phosphorylation and interleukin-2 promoter activation in both resting T-cells and after TCR triggering [99]. Therefore, tonic repression of Lck kinase activity in rafts by Csk seems to set the threshold for TCR signalling and appears necessary to avoid unregulated TCR signalling and T-cell activation. Controlling the Csk kinase activity in rafts may be of major importance to prevent aberrant TCR signalling and immune activation. Together these results suggest the model illustrated in Figure 2. Although not completely understood, this regulation loop involves the transient dissociation of Csk from rafts, allowing Lck to become activated and to perform its catalytic functions, which provides an elegant mechanism for modulation of proximal TCR signalling. Identification and characterization of the unknown PTPase responsible for dephosphorylating Cbp/PAG will undoubtedly be of major importance for understanding the processes controlling the initiation of TCR signalling.

In 1994, we reported that Csk is transiently activated within 1 min after TCR stimulation in T-cells (i.e. upon the time of displacement from rafts), and that, concomitant with this activation, Csk associated with a phosphotyrosine-containing 72–75-kDa protein via its SH2 domain [100]. This protein was recently identified (T. Mustelin, unpublished work), and seems to serve the purpose of anchoring Csk away from lipid rafts, which opens an interesting possibility of Csk shuttling between anchor proteins in the resting and activated T-cells.

Regulation of Csk activity, a molecular mechanism for the inhibitory effect of cAMP on immune functions

Prostaglandin E_2 and other ligands elevating cAMP by binding to G-protein-coupled receptors inhibit TCR-induced T-cell activation and thereby exert important immunoregulatory functions [101]. Based on studies with selective agonists, activation of protein kinase A (PKA) type I ($RI\alpha_2C_2$) is shown to be necessary and sufficient for mediating these effects of cAMP [102,103]. Although PKA can intersect TCR signalling on multiple levels (reviewed in [104]), the observed inhibitory effects of cAMP on TCR-induced ζ-chain phosphorylation point towards an important role for Csk, which is the most upstream PKA target reported so far. We recently showed that PKA, through phosphorylation of Ser-364 in Csk, induces a 2–4-fold increase in phosphotransferase activity of Csk in lipid rafts of T-cells [105].

Analyses of lipid raft purifications from normal resting T-cells for the presence of different subunits of PKA reveal both the catalytic subunit and the regulatory subunit RIα (but no RII subunits) to be constitutively associated with rafts [105]. This suggests that the observed co-localization of PKA type I and TCR in capped T-cells occurs in lipid rafts and that there are mechanisms for specific targeting of PKA type I to these areas involving interaction with an AKAP (A-kinase anchor protein) in lipid rafts (A. Ruppelt and K. Taskén, unpublished work). However, additional possibilities include anchoring of the PKA catalytic subunit, e.g. via the N-terminal myristyl group into rafts, or via interactions with a caveolin-like protein in T-cell rafts, similar to the PKA Cα interaction with caveolin in other cell types [106].

Studies of the organization of G-proteins in the plasma membrane revealed that in addition to G-proteins, the low-density membrane fraction (that most probably represents lipid rafts) contains adenylate cyclase activity [107]. In fact, a substantial fraction of the total isoprenaline- or forskolin-stimulated adenylate cyclase in S49 lymphoma cells was present in

these fractions, strongly suggesting that the receptor–G-protein and G-protein–adenylate cyclase coupling occurs in lipid rafts, and similar data have been obtained for normal T-cells and HEK-293 cells (H. Abrahamsen, T. Vang and K. Taskén, unpublished work). This implies targeting of the molecular machinery necessary for generation of cAMP and activation of PKA type I after engagement of G-protein-coupled receptors to lipid rafts. Figure 3 provides a model for proximal TCR regulation by PKA type I through activation of Csk.

So far, two different mechanisms have been reported to regulate Csk activity. PKA, through phosphorylation of Ser-364, increases Csk kinase activity 2–4-fold, leading to reduced Lck activity and ζ-chain phosphorylation. In addition, Cbp/PAG recruits Csk to the site of action in lipid rafts, and the interaction between the Csk SH2 domain and Cbp/PAG through phosphorylated Tyr-314 (rat Cbp/PAG) also increases Csk activity [108]. Addition of either recombinant Cbp/PAG or peptides corresponding to the Csk SH2-binding site significantly increased Csk kinase activity towards a Src substrate *in vitro*. Thus PKA phosphorylation of Csk, and interaction with Cbp/PAG, may act together in turning on Csk activity, providing a powerful mechanism for terminating activation through receptors eliciting Src kinase signalling.

PTPases INVOLVED IN T-CELL ACTIVATION

Although the molecular mechanisms of signal transduction and T-cell activation have been studied intensely during the past few years (reviewed in [1–4,28,109–115]), the participation of PTPases has largely been neglected, despite the generally accepted notion that they are as important as the PTKs in the control and coordination of the signalling cascades [116–123]. It also seems that PTPases play a crucial role in keeping T-cells in a resting state in the absence of antigen [121–123], as well as in the reversion of activated T-cells back to the resting phenotype upon removal of the activating stimulus [120].

CD45: regulator of Src-family PTKs

The amino acid sequence of the first purified PTPase (PTP1B) unexpectedly revealed that a well-known and abundant transmembrane glycoprotein, the leucocyte common antigen CD45 [124], displayed a high degree of amino acid sequence similarity [125] and was, in fact, a PTPase. As this was published in 1989, we were pursuing a PTPase that activated Lck in T-cell membrane preparations and found that it was CD45 [126]. The following year, M. Thomas' laboratory showed that T-cells lacking CD45 fail to respond to stimulation by antigen or mitogenic antibodies [127]. Together with several subsequent papers showing that CD45 was required for TCR-triggered tyrosine phosphorylation of cellular proteins [128], activation of phospholipase C [128] and calcium mobilization [129], and that loss of CD45 correlated with increased Tyr-505 phosphate on Lck [130], these findings led to our current view [6,110,111,115,117–119] of CD45 as a positive regulator of the Src-family PTKs that mediate TCR signalling (Figure 4) [131–137]. It seems that CD45 maintains these PTKs in a dephosphorylated state ready to participate in signal transmission upon subsequent TCR ligation and that continuous CD45 activity is required for T-cell activation to occur [138]. Contrary to initial expectations, no evidence has emerged for any acute change in CD45 function following TCR triggering, but CD45 isoforms may to a variable extent be regulated via dimerization [139–141]. However, to complicate matters, there are a few papers showing that CD45 can also

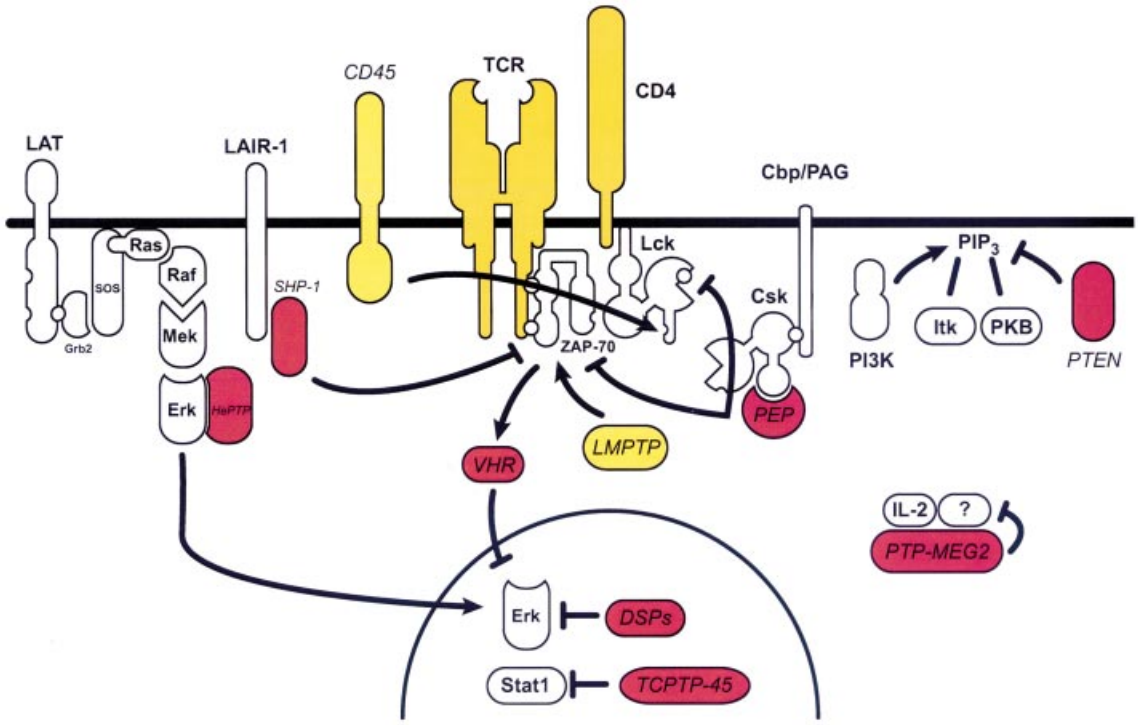

Figure 4 PTPases in T-cell signalling

Schematic illustration of intracellular signalling pathways and kinases involved in T-cell activation as in Figure 1 and depicting roles for PTPs with a defined functional role in T-cell signalling and activation. CD45 and LMPTP dephosphorylate inhibitory residues in Lck and ZAP-70 and have an activating function (indicated by yellow backgrounds) whereas the other PTPases inhibit T-cell signalling at various levels (red backgrounds). PI3K, phosphoinositide 3-kinase; PIP_3, $PtdIns P_3$; IL-2, interleukin-2; DSP, dual-specificity phosphatase; LAIR-1, leucocyte-associated Ig-like receptor-1.

dephosphorylate the positive regulatory site on Src-family PTKs, leading to their inactivation [142], and that CD45 may have additional substrates, such as Jak-family PTKs [143]. New data and ongoing work on the spatiotemporal distribution of CD45 following T-cell activation may elucidate some of the potentially conflicting roles of CD45 as both a positive and a negative regulator of TCR signalling. If CD45 is artificially and constantly targeted to lipid rafts, it acquires a negative regulatory role [144], whereas a fraction (5%) of endogenous CD45 is present in rafts prior to T-cell activation but exits rafts upon activation [145]. Furthermore, examination of supramolecular activation clusters (SMACs) in the contact area of T-cells with antigen-presenting cells indicates that CD45 is present in the central SMACs for a short period of time sufficient to activate Lck to allow phosphorylation of ζ and ZAP-70, but not long enough to give inhibition by dephosphorylation of Lck and its substrates [146]. Thus the positive role of CD45 in TCR signalling is still generally accepted as the foremost function of CD45, but it may well be that this function is counterbalanced by some simultaneous inhibitory functions, all of which appear to relate to the timing of CD45 localization.

Additional PTPases in T-cell activation

Since CD45 has a restricted and mainly positive function in TCR signalling, the PTPases that counteract the PTKs and dephosphorylate the numerous key signalling components must be sought elsewhere. Importantly, the PTKs themselves (with the exception of Csk) are regulated by reversible tyrosine phosphorylation at multiple sites, each with their unique impact on the activity and function of the PTK. Thus, several PTPases are needed just for PTK regulation.

Of the $\approx$ 95 human PTPase genes, at least 30 are known to be expressed in T-cells [116–119,138], including the transmembrane PTPases CD45, CD148, RPTPα and RPTPϵ (the two latter at low levels), the intracellular enzymes PEP, SHP1, SHP2, T-cell PTPase (TCPTP), PTP1B, haematopoietic PTPase (HePTP), PTPase with PEST sequences (PTP-PEST), PTPase from megacaryocytes 2 (PTP-MEG2), PTP-BAS, PTPH1, PTP-MEG1, PTP36, PRL-1, PRL-2 and low-M_r PTPases (LMPTPs) A, B and C, and the dual-specificity PTPases Pac-1, mitogen-activated protein kinase (MAP kinase) phosphatase (MKP) 1, MKP3, MKP6, VH1-related (VHR), VHR-related MKPX (VHX), hYVH1, PTEN and CDC25. Some of these regulate various steps of T-cell activation and proliferation (depicted in Figure 4), while others regulate totally unrelated aspects of T-cell physiology. Indeed, we have found that some PTPases (e.g. TCPTP or PTP-MEG2) do not affect our assays for TCR signalling even when overexpressed up to 100-fold over the endogenous level [147], whereas others inhibit or stimulate responses even when expressed at physiological levels [147–155]. This supports the notion that PTPases are specific and have restricted sets of substrates. The PTPases that participate in T-cell activation are briefly discussed in the following sections.

The Csk-associated PTPases PEP and PTP-PEST

The 110 kDa PEP [156] enzyme is expressed only in haematopoietic cells, including T-cells [143,150], where it resides mostly in the submembranous cytosol close to the plasma membrane [143] (not only in the nucleus, as initially reported [157]), giving it access to substrates at the plasma membrane. Indeed, PEP is a potent negative regulator of TCR signalling with a very receptor-proximal point of action [150,158], most likely related to its tight association with the SH3 domain of Csk [90]. More specifically, it seems that PEP is the PTPase that dephosphorylates Tyr-394 of Lck and Tyr-417 of Fyn concomitantly with the rephosphorylation of the C-terminal negative regulatory sites of these kinases by Csk, explaining the tight linkage between these events in intact T-cells. PEP may also be a regulator of Csk and vice versa.

PTP-PEST is closely related to PEP and also associates with Csk [159]. PTP-PEST is more widely expressed than PEP and appears to play a somewhat different physiological role as a negative regulator of cell adhesion. A prominent substrate for PTP-PEST is the 130 kDa Cas protein [160,161], which is important for focal adhesions. PTP-PEST also dephosphorylates other focal adhesion proteins. Homozygous deletion of the PTP-PEST gene in mice is embryonically lethal [161], suggesting that PTP-PEST is essential. In T-cells, PTP-PEST has been shown to dephosphorylate several signalling proteins that participate in TCR signalling, such as Shc, Pyk2, Fak and Cas, and to counteract the Ras pathway [162]. It is, however, not known if PEP shows any redundancy with PTP-PEST in these tasks.

The SH2 domain-containing PTPases SHP1 and SHP2

The currently best understood intracellular PTPase is SHP1, which is expressed mostly in cells of haematopoietic lineages (reviewed in [117–119]). SHP1 has two SH2 domains in its N-terminal half, while the C-terminal half is a classical PTPase domain. The function of SHP1 is regulated by a class of inhibitory surface receptors, which possess an immunoreceptor tyrosine-based inhibitory motif (ITIM) in their intracellular tails (reviewed in [117–119,163]). In a manner reminiscent of the ITAMs in the CD3 and ζ subunits of the TCR, this motif is first phosphorylated by Lck or Fyn, and then recruits the tandem SH2 domains of SHP1. This binding activates SHP1 and juxtaposes it to its substrates. The biology of ITIM-containing receptors is a new and promising field of study, which is likely to shed more light on the function of SHP1 and the dampening of immune responses.

The homozygous loss of SHP1 leads to the *motheaten* phenotype in mice, which is characterized by spotty hairloss (hence the name) and a number of abnormalities in the function of the immune system and, in particular, in the function of phagocytic leucocytes. Thymocytes from these mice are hyper-responsive to TCR stimulation [164], suggesting that SHP1 plays a role as a negative regulator of signalling during T-cell maturation in the thymus. At least part of this function is mediated through a direct dephosphorylation of the ZAP-70 and Syk PTKs, resulting in decreased downstream signalling [152]. However, the physiological role of SHP1 in T-cells is still unclear, largely because no well-defined ITIM-containing receptors have yet been identified in T-cells or thymocytes. A simple overexpression of SHP1, or its catalytically inactive mutant, has little effect on TCR signalling [152], suggesting that an ITIM-containing receptor is indeed needed.

In contrast to SHP1, the closely related SHP2 does not appear to be a negative regulator of TCR signalling pathways. Quite to the contrary, SHP2 functions as a facilitator of the Ras/Raf/MEK [MAP kinase/extracellular signal-regulated protein kinase (ERK) kinase]/ERK pathway through dephosphorylation of an unknown substrate [165]. SHP2 associates with the Gab2 adapter protein [166], which in turn participates in multimeric signalling complexes [167–169]. Unlike SHP1, deletion of the SHP2 gene is embryonically lethal in mice [170].

LMPTP: a positive regulator of ZAP-70

We have observed that the 18 kDa LMPTP, in contrast to all other intracellular PTPases in T-cells, has a positive effect on TCR-induced transactivation of reporter genes [143]. It appears that the reason for this behaviour is that LMPTP, like CD45,

preferentially dephosphorylates a negative regulatory site in an important PTK, in this case Tyr-292 of ZAP-70 [155]. This site is a binding site for the c-Cbl ubiquitin ligase complex, which negatively regulates TCR signalling by accelerating TCR internalization and degradation and also seems to promote the inactivation of ZAP-70 through dephosphorylation. It has been suggested [171] that c-Cbl associates with PEP. Thus PEP may be transported by c-Cbl to the vicinity of triggered TCRs with its associated Lck, Fyn and ZAP-70 PTKs, resulting in dephosphorylation of these PTKs. The dephosphorylation of Tyr-292 by LMPTP presumably reduces all these inhibitory effects of c-Cbl. Interestingly, LMPTP is itself activated by Lck-mediated phosphorylation [172].

The cytoskeletal PTPase PTPH1

As measured by the ability of transfected PTPases to inhibit TCR signal transduction, PTPH1 is perhaps the most efficient negative regulator of T-cell activation [143,151]. The site of action is receptor-proximal, but as yet undefined. One paper [173] identified the 100 kDa valosin-containing protein as a main substrate for PTPH1. Interestingly, this protein is heavily tyrosine phosphorylated in activated T-cells [174], but its possible role in TCR signalling is unknown. The ability of PTPH1 to inhibit TCR signalling is more likely to be related to the dephosphorylation of a plasma membrane-associated substrate (valosin-containing protein is cytosolic, endoplasmic reticulum-bound and nuclear), since PTPH1 required its N-terminal band 4.1-, ezrin-, radixin- and moesin-homology (FERM) domain for inhibitory activity in TCR signalling assays [151]. The FERM domain was also required for plasma membrane localization, but not for catalytic activity of the enzyme itself.

More phosphatases than kinases control MAP kinases

A large portion of the ≈ 95 PTPase genes in the human genome encode for dual-specificity PTPases with known or suspected preference for members of the MAP kinase family [175]. Since MAP kinases are also dephosphorylated by several classical PTPases and by Ser/Thr phosphatases, there clearly are many more phosphatases than kinases acting on the 11 known MAP kinases [ERKs 1, 2, 3, 5 and 7, c-Jun N-terminal kinases (JNKs) 1, 2 and 3, and p38α, δ and γ; Figure 4]. T-lymphocytes utilize at least seven PTPases that act on MAP kinases (HePTP, Pac-1, MKP1, MKP3, MKP6, VHR and VHX). These enzymes are expressed in different compartments of the cell and at different times of the T-cell activation time course. For example, HePTP and MKP3 are present and exclusively cytosolic in resting T-cells, while Pac-1 and MKP1 are inducible and nuclear enzymes. According to the 'sequential phosphatase model' [175], different PTPases are used in a spatially and temporally ordered manner to control the extent, location and duration of MAP kinase activation. In resting T-cells, ERK is kept cytosolic and inactive (despite a basal MEK activity) by HePTP and MKP3, both of which associate tightly with ERK. Upon TCR triggering and ERK activation by Ras/Raf/MEK, these phosphatases dissociate through incompletely understood mechanisms, and phospho-ERK can escape to the nucleus to carry out many of its biological functions. Some 30–60 min later, Pac-1 mRNA is induced (later still is MKP1) and the protein is synthesized and begins to accumulate in the nucleus, where it dephosphorylates both phosphoamino acids in the activation loop of ERK. This results in complete inactivation of ERK, which shuttles back to the cytosol and reassociates with HePTP and MKP3. This model exemplifies how cells use multiple PTPases to achieve a more refined regulation in space and time of protein phosphorylation. By controlling the expression of these PTPases, the cell can fine-tune the basal activity, peak activity, time course, duration and subcellular location of MAP kinases.

The PTPases that dephosphorylate MAP kinases also differ from each other in how they associate with their targets and which ones they select. HePTP forms a specific high-affinity complex with the MAP kinases ERK and p38 using a 15-amino-acid kinase-interaction motif (KIM) in its N-terminus [148]. In the complex, the catalytic centre of HePTP is juxtaposed to the activation loop of ERK, allowing for a very efficient dephosphorylation [148]. This serves to maintain the bound ERK in an inactive state. Furthermore, the complex is apparently arranged so that ERK (if first activated) can phosphorylate a serine and a threonine in the N-terminus of HePTP adjacent to the KIM [148]. This phosphorylation causes dissociation of the complex, allowing ERK to escape. In addition, a serine residue within the KIM can be phosphorylated by cAMP-dependent kinase, also leading to dissociation of the complex [149]. This represents a potentially important mechanism for cross-talk between the immunosuppressive cAMP/PKA system and the MAP kinases ERK and p38.

Mice homozygous for loss of HePTP [176] display up to 3-fold higher MAP kinase responses than controls. Nevertheless, the overall phenotype of the mice is disappointingly normal, suggesting that hyper-responsive MAP kinases can be well tolerated during embryogenesis and immune system development. Perhaps the many other PTPases involved with MAP kinases play sufficiently redundant roles to compensate for the loss of HePTP. Mice lacking MKP1 [177] also lacked a noticeable phenotype. Most likely, double or triple knock-outs will be required to resolve these issues.

VHR, a small dual-specificity PTPase regulated by ZAP-70

VHR is particularly interesting from a TCR signalling point of view because it accumulates at the T-cell/antigen-presenting-cell contact site, where it is phosphorylated at Tyr-138 by ZAP-70 [178]. This phosphorylation is required for VHR to inhibit the ERK and JNK MAP kinases, giving ZAP-70 an unanticipated control over MAP kinase dephosphorylation, in addition to its role as upstream activator of the Ras/Raf/MEK pathway. VHR is recruited and activated with kinetics that match the gradual inactivation phase of ERK after its early peak. Since ZAP-70 plays differential roles in T-cell subsets and in response to different stimuli, this mechanism may be relevant for the different time courses of MAP kinase activation that have been observed in Th1/Th2 cells and in response to partial agonists.

PTPases with more distal roles in T-cell activation

Two PTPases have distinct roles that come into play only late in T-cell activation: PTP-MEG2 on the cytoplasmic face of the interleukin-2-containing secretory vesicles of activated T-cells [179] and the 45-kDa nuclear isoform of TCPTP, which dephosphorylates tyrosine-phosphorylated Stat1 [180] (Figure 4). PTP-MEG2 appears to regulate the size of secretory vesicles by controlling vesicle fusion. The enzyme itself is regulated by phosphoinositides (T. Mustelin, unpublished work). The action of TCPTP on phospho-Stat1 in the T-cell nucleus is regulated by arginine methylation of Stat1 [180].

CONCLUDING REMARKS: COMPLEX MECHANISMS OPERATE DURING T-CELL ACTIVATION

The completion of the human genome project has made it even more clear than before that the complexity of cell physiology is

enormous. Thus it is safe to assume that a versatile and flexible response like TCR-triggered T-cell activation is likely to involve many more components and much more complex and interdigitating mechanisms than are currently understood; indeed we know the functions of less that 10% of the signalling molecules and less than 0.1% of the *in vivo* protein-phosphorylation reactions that occur. It has already long been evident that the T-cell is not simply a collection of soluble enzymes, but a highly organized molecular machinery, where the spatiotemporal regulation of protein–protein interactions and dynamic protein networks synergize to affect the biological outcome. The role of lipid rafts as platforms for the enrichment of signalling molecules and the exclusion of negative regulators is a good example of how such supramolecular organization may work. Interestingly, it has very recently been found that there are distinct types of lipid microdomain and that 'rafts' may have distinct subdomains or types with different compositions and functions [181]. The dynamic nature of these structures and the rapid movement of molecules in and out of microdomains also complicate their study.

An emerging theme in the concept of lipid rafts in TCR signalling is the transient displacement of negative regulators of signalling, such as PTPases and Csk (Figure 2). Many investigators have proposed that removal of CD45 plays a permissive role in TCR signalling [144–146]. However, the predominantly positive role of CD45 is not readily compatible with this notion, unless its continued presence in SMACs makes CD45 assume a negative role with regard to the substrates of Lck. In addition, it is likely that one or several inhibitory PTPases are actively removed from the vicinity of the triggered TCRs and the associated PTKs and signalling complexes. This would alleviate the strong pressure against tyrosine phosphorylation created by the up to 1000-fold higher PTPase activity than PTK activity in T-cell plasma membranes. There are indications that PEP and PTPH1 may be such excluded enzymes (T. Mustelin, unpublished work), but little has so far been done to address these issues.

The removal of Csk from lipid rafts occurs through acute dephosphorylation of the lipid raft-anchored Csk-binding protein Cbp/PAG [92,99] by an unknown PTPase (possibly PEP). As Csk binds Cbp/PAG via its SH2 domain and PEP via its SH3 domain, these two associations can occur simultaneously. Thus dissociation of Csk from Cbp/PAG in the lipid rafts is probably accompanied by the removal of PEP as well. If PEP is also the PTPase that dephosphorylates Cbp/PAG, then the departure of Csk-PEP will allow Cbp/PAG to be rephosphorylated by Src-family PTKs. This indeed occurs some 5–10 min after TCR triggering [99]. At this time, Csk comes back to the lipid rafts and can again suppress Lck and Fyn.

T-cell activation is not only governed by TCR triggering, but also strongly influenced by a number of other surface receptors that use similar, partially similar or totally different signalling pathways. The area of signalling pathway cross-talk is growing increasingly hot and has already revealed many complex mechanisms that are of great physiological importance. A good example is the above mentioned cross-talk between the immunosuppressive G-protein-coupled receptor/cAMP/PKA pathway and Csk [105], both of which are inhibitory, which involves a Ser/Thr kinase signalling pathway (Figure 3). Interestingly, the cAMP inhibitory pathway is shown to be hyperactivated in T-cells from HIV-infected patients and targeting of the pathway by antagonists reverses T-cell dysfunction in HIV T-cells *ex vivo* [182].

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