CTL activation is induced by cross-linking of TCR/MHC-peptide-CD8/p56^{lck} adducts in rafts

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To investigate the role of the coreceptor CD8 and lipid rafts in cytotoxic T lymphocyte (CTL) activation, we used soluble mono- and multimeric H-2K^d-peptide complexes and cloned S14 CTL specific for a photoreactive derivative of the *Plasmodium berghei* circumsporozoite (PbCS) peptide 252–260 [PbCS(ABA)]. We report that activation of CTL in suspension requires multimeric K^d-PbCS(ABA) complexes co-engaging TCR and CD8. Using TCR ligand photo-cross-linking, we find that monomeric K^d-PbCS(ABA) complexes promote association of TCR/CD3 with CD8/p56^{lck}. Dimerization of these adducts results in activation of p56^{lck} in lipid rafts, where phosphatases are excluded. Additional cross-linking further increases p56^{lck} kinase activity, induces translocation of TCR/CD3 and other signaling molecules to lipid rafts and intracellular calcium mobilization. These events are prevented by blocking Src kinases or CD8 binding to TCR-associated K^d molecules, indicating that CTL activation is initiated by cross-linking of CD8-associated p56^{lck}. They are also inhibited by methyl- β -cyclodextrin, which disrupts rafts and by dipalmitoyl phosphatidylethanolamine, which interferes with TCR signaling. Because efficient association of CD8 and p56^{lck} takes place in rafts, both reagents, though in different ways, impair coupling of p56^{lck} to TCR, thereby inhibiting the initial and essential activation of p56^{lck} induced by cross-linking of engaged TCR.

Key words: CTL / TCR / CD8 / Raft / MHC-peptide

1 Introduction

CD8⁺ cytotoxic T lymphocytes (CTL) recognize antigenic peptides bound to MHC class I molecules on target cells by means of their TCR [1, 2]. Initial TCR engagement activates various auxiliary molecules, such as LFA-1, CD8 and CD2, to bind avidly to their respective ligands, resulting in CTL-target cell conjugate formation and activation of various signaling cascades [1, 2]. Using soluble MHC-peptide complexes for T cell activation, the com-

plexity of T cell signaling can be reduced, permitting stringent analysis of the initial molecular events of TCR signaling. Studies performed on CD4⁺ T cells demonstrated that T cell activation by soluble MHC-peptide complexes requires that these are multimeric, at least dimeric [3–6]. By contrast, Delon et al. [7] reported that soluble monomeric K^d-peptide complexes induce intracellular calcium mobilization in CD8⁺ T cells, concluding that cross-linking of TCR and CD8 by monomeric MHC-peptide is sufficient for activation of CD8⁺ T cells. However, Daniels and Jameson [8] found that mobilization of intracellular calcium in CD8⁺ T cells from TCR transgenic mice was induced by multimeric, but not by monomeric MHC-peptide complexes. Thus, the initial molecular interactions leading to T cell activation by soluble MHC-peptide complexes, in particular the role of the coreceptor, are still poorly understood.

CD8 plays a crucial role in T cell activation and development. By binding to TCR-associated MHC molecules, CD8 greatly strengthens TCR-ligand interaction on cells [9, 10]. Because CD8 can associate with the Src kinase p56^{lck} (lck), as well as with the linker of activation of T

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Abbreviations: **ABA:** 4-Azidobenzoic acid **DIG:** Detergent-insoluble glycolipid complex **DPPE:** Dipalmitoyl phosphatidylethanolamine **DOPE:** Dioleoyl phosphatidylethanolamine **GPI:** Glycosyl phosphatidylinositol **IASA:** Iodo-4-azido-salicylic acid **ITAM:** Immunoreceptor tyrosine-based activation motif **LAT:** Linker of activation of T cells **MCD:** Methyl- β -cyclodextrin **PbCS:** *Plasmodium berghei* circumsporozoite **SH2:** Src homology domain 2

cells (LAT), this coordinate binding recruits these two important signaling molecules to engaged TCR [11–14]. Several aspects of CD8 coreceptor function, however, remain enigmatic. For example, it is well known that the short cytoplasmic tail of CD8 β strengthens the association of lck and LAT [11, 15, 16], but the underlying mechanism is unknown.

The recognition that cell membranes contain detergent-insoluble lipid rafts has important implications for understanding TCR and coreceptor signaling. Rafts are formed by cholesterol, sphingolipids and molecules containing short saturated fatty acids [17, 18]. In the outer leaflet these are mainly glycosyl phosphatidylinositol (GPI)-linked proteins, such as Thy-1 and CD59 [19, 20] and in the inner leaflet palmitoylated and/or myristoylated proteins, e.g. CD4, CD8, lck, p59^{fyn} (fyn) and LAT [21–23]. However, other molecules, such as the abundant phosphatase CD45 are excluded from rafts [24–26]. Thus, by concentrating kinases and their substrates and by excluding phosphatases, rafts constitute privileged sites for phosphorylation reactions and, hence, play a crucial role in T cell activation. Importantly, it has been demonstrated that upon activation of Jurkat cells or thymocytes with anti-CD3 antibodies, TCR/CD3 translocate from the phospholipid membrane fraction to rafts [27, 28]. Other signaling molecules (e.g. ZAP-70, Syk, PLC γ) and adapters (e.g. Grb2 and Vav) follow this trend, at least in part due to activation induced interactions involving Src homology domain 2 (SH2) domains [22, 27–29].

In the present study we examined the role of CD8 and rafts in the activation of cloned CD8⁺ CTL by soluble MHC-peptide complexes. As CTL we used K^d-restricted S14 CTL, which are specific for the *Plasmodium berghei* circumsporozoite (PbCS) peptide 252–260 (SYIPSAEKI) modified with photoreactive 4-azidobenzoic acid (ABA) on PbCS K259 [PbCS(ABA)]. This experimental system offers the opportunity to photo-cross-link TCR with its ligand, K^d-PbCS(ABA), and then to isolate and analyze engaged TCR [9, 10, 30]. We report that monomeric MHC-peptide complexes co-engaging TCR and CD8 promote association of TCR/CD3 with CD8/lck and that cross-linking of these adducts induces the initial and essential activation of lck in rafts.

2 Results

2.1 S14 CTL activation requires multimeric K^d-PbCS(ABA) complexes, co-engaging TCR and CD8

To elucidate the minimal requirement for activation of S14 CTL, we used soluble K^d-PbCS(ABA) complexes. We first assessed binding of mono- and tetrameric K^d-PbCS(ABA) complexes to S14 CTL. S14 CTL were incubated with graded concentrations of phycoerythrin (PE)-labeled tetramer. As shown in Fig. 1A, half-maximal binding was reached at approximately 0.5 nM of tetramer. Nonspecific binding was less than 9%, as assessed by staining with unrelated PE-labeled K^d-Cw3 peptide tetramer. To estimate the binding of K^d-PbCS(ABA) monomer, S14 CTL were incubated with 3.7 nM of PE-labeled K^d-PbCS(ABA) tetramer and graded concentrations of unlabeled monomer and tetramer, respectively. Half-maximal inhibition of the mean fluorescence intensity (MFI) was observed at approximately 0.29 μ M of the monomer and 2.5 nM of the tetramer (Fig. 1B). These results indicate that monomeric and tetrameric K^d-PbCS(ABA) complexes specifically bind to S14 CTL with a binding difference of about 116-fold.

To examine the ability of monomeric and tetrameric K^d-PbCS(ABA) complexes to elicit intracellular calcium mobilization, indo-1-labeled S14 CTL were incubated with saturating concentrations of monomer or tetramer and calcium-dependent fluorescence of indo-1 measured by flow cytometry. As shown in Fig. 1C, S14 CTL exhibited a strong, but transient calcium mobilization upon incubation with tetramer, but not with monomer. This calcium mobilization was dramatically inhibited by Fab' fragments of anti-K^d α 3 mAb SF1–1.1.1, which block binding of CD8 to TCR-associated K^d [9], as well as by the Src kinase inhibitor PP2. By contrast, cytochalasin D and latrunculin, which block cytoskeleton function [31], had no effect (Fig. 1C). Essentially the same findings were obtained with the T1 CTL clone (data not shown), which also recognizes PbCS(ABA), but unlike S14 CTL, in a CD8-independent manner [32]. These results indicate that activation of CTL in suspension by soluble MHC-peptide complexes requires that these are multimeric and co-engage TCR and CD8.

2.2 On resting S14 CTL CD8 is palmitoylated and partitions in rafts

In view of the importance of lipid rafts for TCR signaling, we first assessed the distribution of CD3 ζ , CD8, lck, LAT and Thy-1 in resting S14 CTL in detergent-soluble (M) and insoluble (DIG) membrane fractions. S14 CTL were

Fig. 1. Tetrameric, but not monomeric K^d-SYIPSAEK(ABA)I complexes induce CD8 dependent intracellular calcium mobilization in cloned S14 CTL. (A) S14 CTL were incubated for 60 min at 4°C with graded concentrations of PE-labeled K^d-SYIPSAEK(ABA)I tetramers (circles), or as control, with unrelated PE labeled K^d-Cw3 peptide 172–179 tetramer (squares). (B) Alternatively S14 CTL were incubated likewise with 3.7 nM PE-labeled tetramer and graded concentrations of unlabeled tetramer (filled circles) or monomeric K^d-SYIPSAEK(ABA)I (open circles) and cell-associated PE fluorescence measured by flow cytometry. In (A) and (B) the mean values and standard deviations of two experiments are shown. (C) For calcium flux experiments indo-1-labeled S14 CTL were incubated at 37°C as indicated or for 3 min with K^d-SYIPSAEK(ABA)I monomer (1.16 μM) or tetramer (84 nM) and calcium-dependent fluorescence of indo-1 was assessed by flow cytometry. Tetramer induced calcium mobilization was abolished in the presence of Fab' fragments of anti-K^d mAb SF1–1.1.1 Fab' (SF'), or the Src kinase inhibitor PP2 (15 μM), but not in the presence of the cytoskeleton inhibitors cytochalasin D (CD; 10 μM) and latrunculin (L; 50 nM). One out of five experiments is shown.

lysed in cold Triton X-100 and M and DIG fractions prepared. As assessed by SDS-PAGE and Western blotting, CD3ζ was detected exclusively in the M fraction, whereas, as expected, 80–90 % of the GPI-linked Thy-1 partitioned in DIG (Fig. 2A). Lck partitioned to 40–50% in DIG, CD8 to 10–20 % and LAT to less than 10%. Essentially the same findings were obtained for T1 CTL (data not shown).

Fig. 2. CD8 is palmitoylated and raft-associated. (A) Resting S14 CTL were fractionated in TX-100 soluble M and insoluble DIG fractions and the distribution of CD3ζ, CD8, lck, LAT and Thy-1 was determined by SDS-PAGE and Western blotting. (B) S14 CTL were biosynthetically labeled with [³H]palmitic acid, lysed in Triton X-100, M and DIG fractions prepared and immunoprecipitated with anti-CD8β mAb, resolved on SDS-PAGE (10% reducing conditions), treated or not with hydroxylamine (0.5 M, 6 h at room temperature) and revealed by fluorography. In parallel identical samples were analyzed by Western blotting, using antibodies specific for CD8β and then for LAT. One out of three experiments is shown.

Since protein palmitoylation mediates raft-association of membrane molecules [15, 21, 22, 33, 34], we examined the state of CD8 palmitoylation on resting S14 CTL. As shown in Fig. 2B, [³H]palmitic acid biosynthetically labeled CD8β was found in the DIG, but not the M fraction. The weakly labeled material at 36–38 kDa, was probably co-immunoprecipitated LAT. As assessed by Western blotting, most of LAT and CD8 was in the M fraction, indicating that in resting S14 CTL palmitoylation of these molecules is limited, but determines their raft association. To prove that the observed ³H-labeled materials contained cysteine esterified with radioactive palmitic acid, the gel was treated with hydroxylamine, which hydrolyzes thioesters. After treatment no labeled material was detectable (Fig. 2B), confirming that this was indeed the case.

2.3 Tetrameric, but not monomeric, K^d-PbCS(ABA) complexes induce translocation of TCR/CD3 and signaling molecules to rafts

We next compared the distribution of signaling molecules in resting and activated S14 CTL. S14 CTL were incubated with mono- or tetrameric K^d-PbCS(ABA) complexes and fractionated in M and DIG fractions. The distribution of CD3 ζ , CD8, lck and fyn in M and DIG fractions was determined by SDS-PAGE and Western blotting. Monomeric K^d-PbCS(ABA), even at high concentration (2.3 μ M), failed to induce significant translocation of any of the molecules tested to DIG (Fig. 3), which is consistent with their inability to induce calcium flux (Fig. 1C). By contrast, following incubation with tetramer, a significant fraction of CD3 ζ translocated to DIG. This fraction contained highly phosphorylated pp23 chain. In addition, the fraction of DIG-associated CD8 increased substantially upon incubation with K^d-peptide tetramer; lck, fyn and LAT followed this trend (Fig. 3 and data not shown). Moreover, upon incubation with tetramer, the distribution of Thy-1 remained unchanged (Fig. 3). Importantly, the tetramer induced translocation of CD3 ζ , CD8, lck, and fyn was inhibited by SF1-1.1.1 Fab', demonstrating that CD8 co-engagement is essential for the induction of translocation of signaling molecules to DIG.

Fig. 3. K^d-SYIPSAEK(ABA)I tetramer, but not monomer, induces translocation of TCR/CD3 and palmitoylated molecules to rafts. (A) S14 CTL were incubated at 37°C for 90 s with K^d-SYIPSAEK(ABA)I monomer (1.16 μ M) or tetramer (160 nM) in the absence or presence of anti-K^d α 3 SF1-1.1.1 Fab' (20 μ g/ml). The cells were lysed in cold Triton X-100 and fractionated in M and DIG fractions. Aliquots of the fractions were subjected to SDS-PAGE and Western blotted with antibodies specific for CD3 ζ , CD8 β , lck, fyn and Thy-1.

2.4 Translocation of TCR/CD3 to DIG requires TCR cross-linking and activation of lck

We next examined the cross-linking requirements for lck activation and translocation of TCR/CD3 to DIG. To this end we took advantage of the unique feature of our experimental system, namely that S14 CTL recognize the photo-reactive peptide derivative PbCS(ABA) and hence are amenable to TCR photo-cross-linking by K^d-PbCS(ABA) [30]. In the experiment shown in Fig. 4A, S14

Fig. 4. Translocation of TCR/CD3 to rafts is driven by cross-linking induced activation of lck. (A) S14 CTL, untreated or TCR photo-cross-linked with biotinylated K^d-SYIPSAEK(ABA)I monomer, were incubated as indicated with anti-biotin mAb and anti-mouse IgG and Src kinase inhibitor PP2 (15 μ M). After solubilization in cold Triton X-100, M and DIG fractions were prepared, resolved on SDS-PAGE (12 %) and blotted with antibodies specific for CD3 ζ or phosphotyrosine (pY). The pY blot was stripped and re-blotted with anti-lck antibody. One out of three experiments is shown. (B) S14 CTL either untreated or incubated with either K^d-SYIPSAEK(ABA)I monomer (1.16 μ M) or tetramer (160 nM) were lysed in cold Triton X-100 in the absence (-) or presence (+) of phosphatase inhibitors (PI) and fractionated in M and DIG fractions. Kinase activity of immunoprecipitated lck was assessed using [³²P]ATP and CD3 ζ ITAMc peptide as substrate. Mean values and SD were calculated from two experiments.

CTL were incubated with biotinylated K^d-PbCS(ABA) monomer and photo-cross-linked with TCR by UV irradiation. Dimerization of engaged TCR by anti-biotin antibody significantly increased tyrosine phosphorylation of Ick, yet failed to induce appreciable translocation of TCR/CD3 and Ick to DIG. Further Ick tyrosine phosphorylation occurred when anti-biotin antibody was cross-linked with a secondary antibody. Under these conditions CD3 ζ phosphorylation and translocation to DIG was observed. This was abolished by the Src kinase inhibitor PP2.

To directly assess Ick kinase activity, S14 CTL were incubated with K^d-PbCS(ABA) monomer or tetramer, and then separated in M and DIG fractions, which were analyzed for phosphorylation of ITAMc peptide by immunoprecipitated Ick. As shown in Fig. 4B, Ick activity substantially increased following incubation with tetramer, but not with monomer, and was higher in the M than in the DIG fraction. These results are consistent with the Ick phosphorylation observed in the previous experiment, indicating that the Ick phosphorylation reflects auto-phosphorylation. Remarkably, when no phosphatase inhibitors were present in the lysis buffer, no kinase activity was observed in the M fraction, while it was unaffected in the DIG fraction.

Together these results demonstrate that (i) translocation of TCR/CD3 to DIG requires previous activation of Ick; (ii) Ick is activated by dimeric or, more efficiently, by multimeric cross-linking of CD8-associated Ick, and (iii) Ick activity is quenched by phosphatases in the M, but not the DIG fraction.

2.5 Methyl- β -cyclodextrin and dipalmitoyl lipid inhibit activation of S14 CTL

Based on the observations that CD8, Ick, fyn and LAT partition in rafts, because they are palmitoylated and that this is crucial for their function (Fig. 2 and [15, 21, 22, 35–37]), we examined whether exogenous dipalmitoyl lipid inhibits activation of S14 CTL. The intracellular calcium mobilization in S14 CTL elicited by K^d-PbCS(ABA) tetramer was dramatically impaired by dipalmitoyl phosphatidylethanolamine (DPPE), but not by dioleoyl phosphatidylethanolamine (DOPE) (Fig. 5). DPPE differs from DOPE only by having unsaturated oleic acid in place of saturated palmitic acid and extensively partitions in lipid rafts, whereas DOPE does not [38]. Pretreatment of S14 CTL with DPPE also prevented tetramer-induced translocation of CD3 ζ , CD8 and Ick to rafts [38].

The tetramer-induced intracellular calcium mobilization in S14 CTL was also inhibited by methyl- β -cyclodextrin (MCD), which destabilizes rafts (Fig. 5 and [36]). Together

Fig. 5. MCD and exogenous dipalmitoyl lipid impairs CTL activation. Indo-1-labeled S14 CTL, untreated or pulsed with 7 μ M DPPE, or DOPE or 1 mM MCD were incubated at 37°C for 90 s with K^d-SYIPSAEK(ABA)I tetramer (50 nM) and calcium-dependent fluorescence of indo-1 was measured by flow cytometry.

these findings indicate that activation of S14 CTL by soluble K^d-PbCS(ABA) tetramer requires raft integrity and raft association of palmitoylated molecules, e.g. CD8 and Ick.

2.6 Monomeric K^d-PbCS(ABA) promote association of TCR/CD3 with CD8/Ick

Taking advantage of TCR photo-cross-linking, we examined the recruitment of Ick to TCR engaged by monomeric K^d-PbCS(ABA) and how it was affected by blocking of CD8 and DPPE, respectively. We photo-cross-linked labeled S14 CTL with soluble covalent K^d-[¹²⁵I]iodo-4-azidosalicylic acid (¹²⁵IASA)-YIPSAEK(ABA)I and assessed the distribution of photo-cross-linked TCR-K^d-PbCS(ABA) in M and DIG fractions. As shown in Fig. 6A, a small, but significant fraction of engaged TCR was in the DIG fraction. This fraction apparently is larger than that in experiments in which

A

B

Fig. 6. Monomeric K^d-SYIPSAEK(ABA)I complexes promote association of TCR/CD3 with CD8/lck. (A) S14 CTL, untreated or previously pulsed with DPPE (7 μM) were incubated in the absence or presence of anti-K^d SF1-1.1.1 Fab' (20 μg/ml) with soluble monomeric K^d-¹²⁵IASA-YIPSEK(ABA)I (52 nM) at 37°C for 15 min. After UV irradiation washed cells were lysed in cold Triton X-100, fractionated in M and DIG fractions, immunoprecipitated with anti-TCR mAb H57 and analyzed by SDS-PAGE and phosphorimaging. (B) Alternatively biotinylated monomeric K^d-SYIPSAEK(ABA)I complexes (1.16 μM) were used for TCR ligand photo-cross-linking. The washed cells were lysed in cold Brij78 plus Brij96 and the detergent soluble fraction immunoprecipitated with streptavidin and blotted with antibodies specific for lck, CD8, or CD3ζ. One out of three experiments is shown.

S14 CTL were incubated with non-radioactive K^d-PbCS(ABA) (Fig. 3 and 4). This difference is due mostly to a considerably higher detection sensitivity in the former, compared to the latter experiment. In the presence of SF1-1.1.1 Fab' (Fig. 6A) or anti-CD8β mAb H35-17 (data not shown), the amount of S14 TCR-K^d-PbCS(ABA) complexes was substantially reduced. Conversely, pretreatment of S14 CTL with DPPE had no appreciable effect on the overall efficiency of TCR ligand binding (data not shown).

Alternatively we used higher concentrations of biotinylated monomeric K^d-SIPSAEK(ABA)I complexes for TCR ligand photo-cross-linking, and analyzed the amount of CD8 and lck that was associated with engaged TCR. As shown in Fig. 6B, immunoprecipitates of K^d-peptide

engaged TCR exhibited substantial amounts of CD8 and lck. As expected, this co-immunoprecipitation was impaired when CD8 binding to TCR-associated K^d was blocked by SF1-1.1.1 Fab'. Importantly, when S14 CTL were pretreated with DPPE, the amount of TCR-associated lck, but less of CD8, was considerably reduced. These results indicate that SF1-1.1.1 Fab' and DPPE inhibit recruitment of lck to K^d-peptide engaged TCR, which is consistent with the finding that these reagents block CTL activation (Fig. 1 and 5).

3 Discussion

The present study demonstrates that activation of CD8⁺ CTL in suspension by soluble MHC-peptide complexes requires that these are multimeric and co-engage TCR and CD8. Monomeric K^d-PbCS(ABA) complexes failed to activate CTL (*i.e.* calcium mobilization, lck phosphorylation and translocation of signaling molecules to rafts), regardless of whether they are CD8 dependent or not (Fig. 1 and unpublished data). Our results are in agreement with other studies showing that dimeric [4, 5] and tetrameric [3, 8], but not monomeric [3, 6, 8], MHC-peptide complexes activate CD4⁺ and CD8⁺ T cells. However, our findings are at odds with a study by Delon et al. [7] reporting that CD8⁺ T cells are activated by monomeric K^d-peptide complexes. We demonstrate here that monomeric K^d-PbCS(ABA) complexes recruit CD8/lck to TCR/CD3, as they suggested, but that for T cell activation CD8-associated lck needs to be activated first (Fig. 4).

Calcium mobilization in S14 CTL elicited by soluble K^d-PbCS(ABA) tetramer was not inhibited by cytoskeleton inhibitors, which abolish calcium flux when CTL are activated with peptide pulsed APC (Fig. 1, unpublished data and [38]). While cell polarization clearly plays an important role in physiological T cell activation, it is not involved in activation of CTL in suspension by soluble MHC-peptide complexes, which provides a unique opportunity to investigate the initial steps of TCR- and CD8-mediated T cell activation in the absence of other molecular interactions.

The proportion of LAT and CD8 associated with Triton X-100 insoluble DIG is much smaller, as has been reported for Jurkat cells or T cell hybridomas (Fig. 2 and [15, 22]). It has been shown that CD4 [21], CD8 (Fig. 2B and [15]), LAT [22], lck [33, 35] and fyn [34] partition in DIG because they are palmitoylated and that this is crucial for T cell activation [17, 39]. The present study demonstrates that CD8 is palmitoylated on normal CTL and that raft-associated CD8 is palmitoylated, whereas CD8 in the Triton X-100 soluble fraction is not (Fig. 2B). This strongly

suggests that the cell-specific differences in raft-association of CD8 and LAT are accounted for by differences in the extent of protein palmitoylation, which is a reversible post-translational protein modification [34, 37, 39].

Studies on Jurkat cells [22, 28, 29] and thymocytes [27] indicated that anti-CD3 antibodies induce translocation of TCR/CD3 and other signaling molecules to rafts. Physiologically antigen-specific activation of T cells is induced by MHC-peptide complexes, which unlike anti-CD3 antibodies, engage not only TCR/CD3, but also the coreceptor (Fig. 1 and 6). We, therefore, used soluble K^d-PbCS(ABA) complexes to investigate the role of CD8 in CTL activation. Taking advantage of TCR ligand photo-cross-linking, we were able to demonstrate, for the first time, that the initial and essential lck activation precedes translocation of TCR/CD3 to DIG and is induced by K^d-peptide mediated cross-linking of CD8/lck in rafts (Fig. 4–6). Since CD8 associates with lck, its cross-linking by coordinate binding to TCR-associated multimeric K^d-peptide complexes results in cross-linking of lck (Fig. 4 and [12, 15]). This, as has been shown previously for anti-CD8 antibody [11, 15, 40], activates lck, because this kinase is activated by trans-phosphorylation of tyrosine 394 in the regulatory A loop, irrespectively of the state of phosphorylation of the regulatory tyrosine 505 [40].

Once lck activity and hence phosphorylation of CD3 ITAM exceeds a critical threshold, ZAP-70 as well as other molecules with SH2 domains, such as Syk and fyn, are recruited to TCR/CD3 (Fig. 3, and [1, 2, 41]). Subsequent phosphorylation of ZAP-70 by lck induces its kinase activity and promotes binding of lck to ZAP-70, thus strengthening CD8/lck association with TCR/CD3 [41, 42]. Also LAT gets firmly recruited to TCR/CD3, which is crucial, because LAT, upon phosphorylation by ZAP-70, recruits various adapter and signaling molecules to TCR/CD3, thus linking the initial TCR activation to diverse down-stream signaling cascades [22, 29, 43, 44]. It has been reported that LAT, by associating with the coreceptor, is recruited to engaged TCR, the same way as lck [13, 14]. Consistent with this is our finding that LAT is weakly co-immunoprecipitated with CD8 (Fig. 2B). However, the observations that the content of LAT and TCR/CD3 increases considerably in rafts upon activation of CTL with tetramer (unpublished results) or of Jurkat cells with anti-CD3 antibody [22, 28, 29], suggests that there exists an additional, activation dependent recruitment of LAT to engaged TCR/CD3.

The findings that mutation or deletion of the palmitoylation sites of LAT, lck, fyn and CD8 [11, 15, 16, 22, 23], inhibition of protein palmitoylation by 2-bromopalmitate

or unsaturated fatty acids [39, 45], disruption of rafts by MCD [36] or inhibition of recruitment of palmitoylated molecules to rafts by DPPE (Fig. 5 and 6 and [38]) have deleterious effects on TCR-mediated cell activation stresses the importance of rafts and raft association of palmitoylated signaling molecules for TCR signaling. Furthermore, the observation that the high phosphoform of CD3 ζ (pp23) and lck were found in rafts (Fig. 3, 4 and [15, 27]) demonstrates that kinase-mediated phosphorylation reactions take place in rafts, from which the abundant phosphatase CD45 is excluded [24–26]. The finding that lck-mediated phosphorylation is quenched by phosphatases in the M, but not DIG fractions, directly proves this (Fig. 4B).

Our TCR ligand photo-cross-linking experiments demonstrate that coordinate binding of CD8 to TCR-associated K^d molecules strengthens TCR-ligand binding and promotes association of TCR with CD8 (Fig. 6 and [9, 10]). These experiments also show that DPPE substantially reduces the amount of TCR-associated lck. Since CD8 associates with lck predominantly in rafts [15], this indicates that K^d-peptide promote association of TCR/CD3 with CD8/lck in rafts. Since activation of S14 CTL by K^d-peptide requires that these are multimeric and co-engage TCR and CD8, the initial and essential lck activation comes from cross-linking of raft-associated adducts of CD8/lck with TCR/CD3 (Fig. 1, 4 and 6).

4 Materials and methods

4.1 Cells, antibodies, immunoprecipitation and Western blotting

The CTL clones S14 and T1 were generated and propagated as described previously [30]. The following antibodies were obtained from American Type Culture Collection (Manassas, VA): anti-CD8 KT-112 and H35–17, anti-CD8 53.6.72, anti-CD3 ζ H-146, anti-K^d α 3 SF1–1.1.1 and anti-TCR H-57. Anti-lck antibody 3A5, anti-LAT and anti-phosphotyrosine 4G10 antibodies were from Upstate Biotechnology. Anti-biotin antibody BN 34, rabbit anti-rat IgG and goat anti-mouse IgG were from Sigma (Buchs, Switzerland). Anti-fyn antiserum was a generous gift from Dr. White (Harvard Medical School, MA) and anti-lck antiserum from Drs. Acuto and Di Bartolo (Pasteur Institute, Paris, France). Conditions for immunoprecipitation and Western blotting have been described previously [10, 27]. In brief immunoprecipitations were performed overnight at 4°C using mAb absorbed on protein A or protein G Sepharose (Pharmacia, Uppsala, Sweden). The beads were washed twice in lysis buffer containing half the detergent concentrations (see Sect. 4.4) For CD8 immunoprecipitations no EDTA and EGTA was used unless specified otherwise. SDS-PAGE was performed on linear gels 10% or 12% under reducing conditions. For Western blotting 7% of each fraction was analyzed.

4.2 Soluble K^d-PbCS(ABA) complexes

Monomeric covalent K^d-¹²⁵IASA-YIPSAEK(ABA)I complexes were obtained by incubating soluble K^d expressed in CHO cells with radioactive ¹²⁵IASA-YIPSAEK(ABA)I and selective photo-activation of the IASA group by UV irradiation at 350 nm as described [9, 10, 30]. The photo-cross-linked K^d-¹²⁵IASA-YIPSAEK(ABA)I complexes were purified by FPLC gel filtration and their specific radioactivity was about 2,000 Ci/mmol. To prepare larger amounts of K^d-PbCS(ABA) complexes K^d heavy chain and human β 2 microglobulin, expressed in *E. coli*, were refolded using the dilution method [46, 47]. As peptide SYIPSAEK(ABA)I, rather than IASA-YIPSAEK(ABA)I, was used due to substantially higher refolding efficiency refolded monomer were biotinylated and purified by MonoQ and gel filtration chromatography as described [46, 47]. Tetramers were formed and purified as described [3, 46, 47], using avidin or streptavidin-PE (Molecular Probes, OR) and a Superdex 200 column (Pharmacia, Uppsala, Sweden) for gel filtration.

4.3 K^d and TCR photo-cross-linking

For TCR photo-affinity labeling, 10⁷ S14 CTL were resuspended in 1 ml of serum-free DMEM and incubated with 10⁷ cpm of ¹²⁵IASA-YIPSAEK(ABA)I or 30 μ g K^d-PbCS(ABA) at 37°C for 15 min or for 30 min at room temperature. Following UV irradiation for 40 s using a 90-W mercury fluorescence lamp emitting at 312 $\pm$ 40 nm (BioBlock, Illkirch, France), the cells were washed twice with PBS and fractionated as described below.

4.4 CTL activation, DIG fractionation and labeling with [³H]palmitate

Unless stated otherwise, CTL were pulsed with K^d-PbCS(ABA) monomer (1.16 μ M) or tetramer (0.16 μ M) for 2–3 min at 37°C. For TCR photo-cross-linking, S14 CTL were incubated with monomer for 1 h at 4°C and photo-cross-linked by UV irradiation as described [9]. Cells (5 $\times$ 10⁷) were lysed on ice for 20 min in 1 ml of 1% Triton X-100 in MNE buffer (25 mM MES, 150 mM NaCl, 5 mM EDTA; pH 6.5) containing 0.2 mM sodium ortho-vanadate, 15 mM sodium pyrophosphate, 10 mM glycerophosphate, 5 μ g/ml leupeptin, 5 μ g/ml pepstatin and 100 μ M PMSF. After douncing (10 strokes), the lysate was spun at 500 $\times$ g for 7 min at 4°C, the nuclear pellet was washed sequentially with 0.3 ml Triton X-100 in MNE buffer and 0.3 ml DIG solubilization buffer (50 mM octyl-glucoside in 20 mM Tris, 200 mM NaCl, 2 mM EDTA, 2 mM EGTA; pH 8.0). The post-nuclear supernatant was centrifuged at 100,000 $\times$ g for 60 min at 4°C. The remaining supernatant is referred to as phospholipid membrane (M) fraction. The resulting pellet was solubilized on ice with 1 ml DIG solubilization buffer, to give the DIG fraction. Cloned CTL were labeled with [³H]palmitate (NEN, Boston, MA) as described previously [22].

4.5 Intracellular calcium mobilization and lipid treatment

Cells were incubated with indo-1/AM (Sigma, 5 μ M, 10⁶ cell/ml) at 37°C for 45 min. After washing with DMEM, indo-1-labeled cells were incubated with 84 nM tetrameric or 1.16 μ M monomeric K^d-PbCS(ABA) complexes at 37°C as indicated. Changes in intracellular free calcium concentrations were measured at 37°C using a FACStar cytofluorometer (Becton Dickinson, Erembodegen, Belgium). For treatment with PP2 (Calbiochem, CA) S14 CTL were pre-incubated for 45 min at 37°C with indo-1 and PP2 (15 μ M) in DMEM containing 5% FCS. For treatment with lipids S14 CTL were incubated at 37°C for 45 min in DMEM containing 7 μ M DPPE or DOPE (Sigma). For treatment with MCD, S14 CTL were incubated for 15 min at ambient temperature with 1 mM MCD (Sigma).

4.6 In vitro kinase assay

The assay was performed as described previously [48] using a biotinylated peptide corresponding to ITAMc of CD3 ζ . Following incubation, the peptide was immunoprecipitated with streptavidin-Sepharose and quantified by SDS-PAGE (20%, reducing conditions) and phosphoimaging using a PhosphorImager and the ImageQuant software (Molecular Dynamics, Inc, Sunnyvale, CA).

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