Engagement of T cell receptor triggers its recruitment to low-density detergent-insoluble membrane domains

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T-cell receptors (TCRs) upon binding to peptide–MHC ligands transduce signals in T lymphocytes. Tyrosine phosphorylations in the cytoplasmic domains of the CD3 ($\gamma\delta\epsilon$) and ζ subunits of the TCR complex by Src family kinases initiate the signaling cascades via docking and activation of ZAP-70 kinase and other signaling components. We examined the role of the low-density detergent-insoluble membranes (DIMs) in TCR signaling. Using mouse thymocytes as a model, we characterized the structural organization of DIMs in detail. We then demonstrated that TCR engagement triggered an immediate increase in the amount of TCR/CD3 present in DIMs, which directly involves the engaged receptor complexes. TCR/CD3 recruitment is accompanied by the accumulation of a series of prominent tyrosine-phosphorylated substrates and by an increase of the Lck activity in DIMs. Upon TCR stimulation, the DIM-associated receptor complexes are highly enriched in the hyperphosphorylated p23 ζ chains, contain most of the TCR/CD3-associated, phosphorylation-activated ZAP-70 kinases and seem to integrate into higher order, multiple tyrosine-phosphorylated substrate-containing protein complexes. The TCR/CD3 recruitment was found to depend on the activity of Src family kinases. We thus provide the first demonstration of recruitment of TCR/CD3 to DIMs upon receptor stimulation and propose it as a mechanism whereby TCR engagement is coupled to downstream signaling cascades.

Keywords: detergent insoluble/membrane domain/ signaling/TCR/tyrosine phosphorylation

Introduction

The plasma membrane lipid bilayer contains membrane domains with distinct compositions and functions (Edidin, 1992; Sheetz, 1993). Among them, the sphingolipid-cholesterol rafts, also known as GEMs (glycolipid-enriched membrane domains), DIGs (detergent-insoluble glycolipid-enriched domains) or DRMs (detergent-resist-

ant membranes) (Brown and London, 1997; Simons and Ikonen, 1997) have been proposed to be involved in membrane trafficking, cell morphogenesis and signal transduction mechanisms (Harder and Simons, 1997; Simons and Ikonen, 1997). A variety of signaling molecules are concentrated in raft domains, including Src family kinases, heterotrimeric and Ras-like G proteins, as well as molecules involved in Ca^{2+} influx (Simons and Ikonen, 1997). Rafts can be isolated as low-density, Triton X-100-insoluble membrane complexes that are enriched in sphingolipids, cholesterol and glycosylphosphatidylinositol (GPI)-anchored proteins (Brown and Rose, 1992). Studies from model systems suggest that rafts represent 'liquid ordered' phases with a reduced membrane fluidity (Brown and London, 1997; Hooper, 1998). Although biochemical data have characterized these structures in a wide variety of cells (Brown and London, 1997; Simons and Ikonen, 1997), scant information is available currently on their *in situ* detection, most likely due to the structural dynamics of rafts in the membrane (Harder and Simons, 1997; Hooper, 1998) and to the potential alterations induced by probe binding and sample fixation procedures (Mayor *et al.*, 1994; Smart *et al.*, 1996; Fujimoto *et al.*, 1997; Wu *et al.*, 1997). However, the existence of rafts *in situ* is strongly supported by the clustered distribution of GPI-anchored proteins and glycosphingolipids (GSLs), recently visualized under more appropriate fixation conditions (Danielsen and van Deurs, 1995; Stauffer and Meyer, 1997; Wu *et al.*, 1997), and their confined diffusion in the plasma membrane (Sheets *et al.*, 1997). In addition, caveolae, originally defined as flask-shaped 60 nm invaginations of the plasma membrane found in some cells, are related to raft domains in terms of composition and/or topography (Anderson, 1993; Schnitzer *et al.*, 1995), although the precise structural relationship currently is debated (Anderson, 1993; Sargiacomo *et al.*, 1993; Schnitzer *et al.*, 1995; Stan *et al.*, 1997). It has been shown that raft domains could be detected as caveolae-independent structures, in cells that both contain or are devoid of caveolae (Brown and London, 1997; Simons and Ikonen, 1997). In cells lacking caveolae, such as lymphocytes and certain neuronal cells, the concentration of sphingolipids, cholesterol, GPI-anchored proteins and signaling molecules (e.g. Src family kinases) in rafts has been clearly demonstrated (Brown and London, 1997; Simons and Ikonen, 1997).

Engagement of the T-cell receptor (TCR) with its specific peptide–MHC ligand is required for T-lymphocyte activation and differentiation (Malissen and Schmitt-verhulst, 1993; Weiss and Littman, 1994). The TCR is composed of a clonally specific $\alpha\beta$ heterodimer non-covalently associated with the invariant CD3 ($\gamma\delta\epsilon$) complex and ζ homodimer (Weiss and Littman, 1994). The cytoplasmic domain of each CD3 subunit contains either

one ($\gamma\delta\epsilon$) or three (ζ) immunoreceptor tyrosine-based activation motifs (ITAMs) (Wegener *et al.*, 1992; Weiss and Littman, 1994). The earliest signaling event after TCR engagement is the phosphorylation of ITAM tyrosine residues (Malissen and Schmitt-verhulst, 1993; Weiss and Littman, 1994), which is essential in coupling the TCR to the downstream signaling pathways, including hydrolysis of inositol-containing phospholipids, Ca^{2+} mobilization and activation of the Ras/MAPK cascade (Cantrell, 1996). Lck (a Src family kinase) mediates ITAM phosphorylation, with the contribution in certain circumstances of Fyn, another Src kinase (Cantrell, 1996; van Oers *et al.*, 1996). The phosphorylated ITAMs permit ZAP-70 (a Syk family kinase) recruitment and its activation via both a self- and a Lck-dependent phosphorylation mechanism (Qian and Weiss, 1997). ZAP-70 phosphorylates substrates which in turn lead to the subsequent docking and activation of other Src homology 2 (SH2)-containing molecules involved in the amplification and diversification of TCR-initiated signaling. The recently cloned LAT (linker for activation of T cells) (Zhang *et al.*, 1998), a substrate of activated ZAP-70, appears to be a central anchoring molecule for recruitment of multiple signaling molecules. The CD4 and CD8 co-receptor molecules are generally thought to contribute to ITAM phosphorylation by interacting with TCR-bound peptide-MHC ligand, thereby providing CD4/CD8-associated Lck to the TCR/CD3 complexes (Weiss and Littman, 1994). Several recent studies, however, indicate that one of the important roles of Lck-associated co-receptors seems to be to stabilize the otherwise transient TCR-peptide-MHC interaction (Luescher *et al.*, 1995; Hampl *et al.*, 1997). High order oligomerization of TCR, co-receptors and accessory molecules at the adhesion patches facing the antigen-presenting cells (APCs) recently has been proposed to be a key event for initiating effective TCR-mediated signaling cascades; indeed, the formation of these signaling aggregates allows sustained TCR-peptide-MHC interaction, concentrates second messengers and adaptor molecules and excludes negative regulators such as CD45 protein tyrosine phosphatase (PTP) (Germain, 1997; Shaw and Dustin, 1997).

Compartmentalization through protein-protein or protein-lipid interactions has now been recognized as a fundamental mechanism for efficient and organized cell signaling (Liu *et al.*, 1997; Pawson and Scott, 1997). Recent data indicate that dual acylation of Lck at its N-terminus is essential for coupling TCR to downstream signaling pathways, most likely by targeting Lck to the plasma membrane, and in detergent-resistant domains (Kabouridis *et al.*, 1997). Biochemical analysis or direct monitoring in living cells have documented the compartmentalized IgE receptor (Fc ϵ RI) activation within membrane domains that are enriched in GSLs and GPI-anchored proteins, and resistant to detergent extraction (Field *et al.*, 1997; Stauffer and Meyer, 1997). Field *et al.* (1997) observed that aggregation of Fc ϵ RI provokes its rapid and efficient recruitment to these membrane compartments in RBL-2H3 mucosal mast cells, followed by the Src kinase Lyn-dependent phosphorylation of ITAMs in β and γ subunits, the earliest event in the Fc ϵ RI signaling cascade. In the present study, we have explored the role of detergent-resistant membrane compartments that we call DIMs in TCR-mediated signal transduction in mouse thymocytes,

using Brij 58 as a detergent. Our data suggest that DIM-dependent compartmentalized signaling occurs for the TCR, which utilizes, as do other multichain immune receptors, Src family kinases for the initiation of the signaling cascades.

Results

Structural characterization of DIMs in mouse thymocytes

Previous studies have reported the presence of detergent-resistant membrane domains in T-cell lines (Fra *et al.*, 1994; Cerny *et al.*, 1996; Parolini *et al.*, 1996; Rodgers and Rose, 1996) and in all likelihood in thymocytes (Thomas and Samelson, 1992). As Brij 58 seemed to preserve these membrane complexes better in T cells (Bohuslav *et al.*, 1993), we have used this detergent to solubilize thymocyte membranes. DIMs thus refer here to the membrane complexes that are insoluble in Brij 58 at 4°C and float up in a sucrose density gradient.

Figure 1A shows that a part of the surface-biotinylated proteins were recovered as molecular complexes from the buoyant fractions (fractions 2–4/5) separately from the bulk of solubilized materials located at the bottom of the gradient (fractions 8 and 9). These molecular complexes have a density (1.038–1.051/1.063 g/cm³) corresponding to that of previously characterized DIMs (Brown and Rose, 1992; Garcia *et al.*, 1993; Field *et al.*, 1995) and contain a subset of surface proteins with a specific profile, as shown by their enrichment in some but deficiency in other biotinylated proteins. The 25–27 and 18 kDa major proteins enriched in these complexes were identified by immunoprecipitation (see below) and Western blotting (Figure 1B) as the GPI-anchored Thy-1 and ThB molecules, respectively. These complexes exhibit a concomitant enrichment in dual-acylated Src kinases (Fyn and Lck), as well as an exclusion of transferrin receptor (TfR), Rab5 and α -tubulin, representative of transmembrane, farnesylated and cytoskeleton proteins, respectively (Figure 1B and C). Moreover, they were found to contain glycolipids, such as gangliosides (Figure 2A) including GM1 and GD1a (Figure 2C) as well as the mono- and lactocerebrosides (Figure 2B). Thus, we concluded that the molecular complexes partitioning in the buoyant fractions of sucrose gradient were 'true' DIMs, a notion further supported by their solubilization in Brij 58 at 4°C following 0.2% saponin pre-treatment (Figure 1C) or in cold *N*-octylglucoside (data not shown). Saponin is a cholesterol-depleting agent which destabilizes DIM structures rendering them sensitive to detergents such as Triton X-100 or Brij 58 (Cerney *et al.*, 1993; Draberova *et al.*, 1996; Schroeder *et al.*, 1998), whereas *N*-octylglucoside is a direct DIM-dissociating detergent (Brown and Rose, 1992). We also identified the high molecular weight (180–190 kDa) major surface protein weakly partitioning into DIMs (Figure 1A) as CD45 (not shown). Such an observation is consistent with those describing the presence of CD45 in DIMs of human T lymphocytes and murine T lymphoma cells (Parolini *et al.*, 1996; Ilangumaran *et al.*, 1997), but are at variance with the report by Rodgers and Rose (1996), showing that CD45 is excluded from DIMs in the Jurkat T-cell line. However, both studies showed

Fig. 1. Protein distribution in DIMs of mouse thymocytes. **(A)** Analysis of surface-biotinylated proteins in DIM and H fractions. A PNS preparation of biotinylated mouse thymocytes (2×10^8) was treated with Brij 58 at 4°C and fractionated on a sucrose density gradient as described in Materials and methods. Protein contents in 1/20 of each fraction were resolved on 5–15% SDS–PAGE, transferred to PVDF membranes and stained using the streptavidin–HRP/ECL system. The arrows indicate the position of GPI-anchored proteins, Thy-1 and ThB, whereas the arrowheads indicate that of three DIM-excluded unknown surface proteins. **(B)** Immunoblotting analysis of protein distribution in DIM and H fractions. Protein patterns in 1/20 of each fraction of the sucrose density gradient were analyzed on immunoblots stained with the corresponding specific antibodies, as indicated. **(C)** Effect of saponin pre-treatment on DIM sensitivity to Brij 58. The sensitivity of DIMs to Brij 58 was monitored by the distribution of Lck in 1/20 of each fraction over the density gradient. Thymocytes were pre-treated with or without 0.2% saponin for 10 min at 4°C . PNS preparations were treated with Brij 58 and fractionated as described above.

the dramatic down-regulation of CD45 in DIMs as compared with the heavy (H) fraction.

DIMs are not contaminated *in vitro* by heterologous membrane proteins

In order to assess the interaction between the DIM components accurately, we found it crucial to evaluate a possible contamination of DIMs during detergent extraction. To this end, thymocytes from Thy-1.1 and Thy-1.2 congenic mice were mixed together before being subjected to sonication and Brij 58 treatment. DIMs were isolated by a sucrose gradient, immunoprecipitated with Thy-1.1- or Thy-1.2-specific monoclonal antibodies (mAbs) and subsequently blotted with a Thy-1.1-specific mAb. Figure 3 shows that immunoblotting with a Thy-1.1-specific mAb revealed a signal in the Thy-1.1, but not in the Thy-1.2 precipitates. As a control, the Thy-1.2-specific mAb efficiently immunoprecipitated DIMs isolated from Thy-1.2 mice (data not shown). Thus, these results demonstrated that Thy-1.2-containing DIMs were not contaminated by either soluble or DIM-associated Thy-1.1 molecules. Considering that the detergent was added to the cell sonicate, our study indicated that during detergent extraction, DIM vesicles did not artifactually trap soluble material nor did they undergo fusions with heterologous membrane fragments, including DIMs, of the same or different cellular origin. However, these observations did not rule out the possibility that individual DIMs can coalesce to form larger DIM vesicles in the same membrane plane during the detergent extraction.

Co-distribution of GPI-anchored proteins and GSLs (GM1) within DIM vesicles

Figure 4A shows that GPI-anchored molecules and GM1 can be co-immunoprecipitated either by mAbs to individual GPI-anchored proteins (Thy-1, ThB and HSA) or by cholera toxin B subunit (CTB), a GM1-binding molecule, from the buoyant DIM fractions, but not from the H fraction (not shown) of the gradient. The fact that a similar ratio between the different GPI-anchored proteins and GM1 was observed regardless of the probe used for immunoprecipitation is strongly indicative that the same DIMs were isolated via different GPI-anchored proteins or GM1. The more efficient precipitation of DIMs by Thy-1 mAb presumably is due to the abundant Thy-1 content in these membrane vesicles. In the context of a recent report of the topological separation between caveolae enriched in GM1 and the GPI-anchored protein microdomains in endothelial cell membrane (Schnitzer *et al.*, 1995), we have performed an immunogold electron microscopy (EM) study of DIM vesicles labeled with both Thy-1-specific mAb and the GM1-specific CTB. In a previous study (Thimonier *et al.*, 1997), we showed that DIMs isolated from mouse thymocytes appeared as a homogeneous population of vesicles free of contents, delimited by an apparent bilayer and exhibiting a mean diameter of 184 ± 76 nm. In single immunogold labeling experiments (Figure 4B, b and c; Table I), 88 and 84% of total vesicles were labeled homogeneously by Thy-1- or GM1-specific probes, with a mean diameter for the positive vesicles of 198 ± 78 nm and 186 ± 55 nm, respectively. In double

Fig. 2. Glycolipid analysis in DIMs and total membranes of mouse thymocytes. Lipids were extracted from DIM fractions and from microsomal preparations and subjected to HPTLC as described in Materials and methods. (A) Gangliosides extracted from thymocytes (6.6×10^8 cells, lane 5) or from isolated DIMs (equivalent to 1.3×10^9 cells, lane 4) were revealed by resorcinol. Migration of purified standards (lane 1–3) is indicated on the left. The blob due to sucrose in lane 4 is also indicated. (B) Neutral glycolipids extracted from thymocytes (6.6×10^8 cells, lane 3) or from isolated DIMs (equivalent to 1.3×10^9 cells, lane 2) were detected by orcinol reagent. Migration standards (lanes 1 and 4) are indicated on the right: CMH, ceramide monohexoside or cerebroside; CDH, ceramide dihexoside; CTH, ceramide trihexoside. (C) GM1 and GD1a present in 6.6×10^8 thymocytes (lane 3) or in DIMs obtained from an equivalent cell number were detected specifically by CTB overlay, as described in Materials and methods. Migration of purified GM1 (lane 1) or gangliosides purified from mouse optical lobe (lane 4), revealed by CTB, are indicated on the right.

Fig. 3. Lack of contamination of DIMs by heterologous membrane proteins *in vitro*. One-fifth of DIM fractions isolated from Thy-1.1-expressing thymocytes (2.5×10^8) or a mixture of Thy-1.1- and Thy-1.2-expressing thymocytes (1.25×10^8 , each) was immunoprecipitated with the specific mAbs indicated. Immunoprecipitates subsequently were blotted with a Thy-1.1-specific mAb (OX-7).

labeling experiments (Figure 4B,d), double-positive vesicles showed a similar size distribution (mean diameter 209 ± 83 nm), but were less abundant (51%) than predicted by the results of single-labeling experiments. The decrease in the percentage of GM1-positive vesicles in double-labeling versus single-labeling experiments (see Table I) could account for this reduction, most likely due to steric hindrance between the two probes. These results indicated that our isolation procedure yields a population of vesicles with a right-side out orientation within which Thy-1 and GM1 are detected in close association. Thus,

we conclude that GPI-anchored proteins and GSLs exhibit co-distribution within DIMs of mouse thymocytes.

TCR engagement promotes association of TCR/CD3 complexes with DIMs

We observed that a small fraction of the TCR ζ chains was present in the light gradient fractions in non-stimulated mouse thymocytes and that the amount of TCR ζ in this fraction significantly increased immediately following TCR/CD3 cross-linking by an anti-CD3 ϵ mAb (Figure 5A). This result suggested that a recruitment of TCR ζ to these buoyant molecular complexes is occurring upon TCR engagement. The TCR ζ -containing membrane complexes appeared to be a subset of DIMs, inasmuch as they were fully solubilized in cold Brij 58 upon cholesterol depletion; moreover, they could be isolated by mAb specific for GPI-anchored proteins with the same efficiency as the bulk of DIM vesicles (see below). The TCR-triggered recruitment of TCR ζ chains to DIMs was selective since a similar DIM versus H fraction sorting for ThB (Figure 5A) and Lck (Figure 5C) was maintained after TCR stimulation. The recruitment is dependent on TCR signaling as shown by the failure to detect changes in the amount of TCR ζ associated with DIMs upon thymocyte incubation with an anti-Fas mAb (data not shown). In addition, TCR ζ recruitment to DIMs could also be observed when TCRs were physiologically stimulated with an appropriate peptide–MHC complex. These results were obtained using thymocytes from female transgenic mice that expressed a transgenic TCR recognizing the male H-Y antigen presented by MHC class I molecule H-2D^b (Swat *et al.*, 1991). Figure 5B shows enhanced association of TCR ζ with DIMs when thymocytes from female H-Y TCR transgenic mice were incubated with APCs from C57BL/6 male mice that carry H-Y peptide bound to MHC, as compared to that observed with APCs from C57BL/6 female mice.

These results prompted us to examine the recruitment of the full TCR/CD3 complex to DIMs following TCR/CD3 ligation. As illustrated in Figure 5C, in mouse thymocytes, both TCR $\alpha\beta$, ζ and CD3 ϵ chains were found to be recruited to DIMs upon TCR engagement. Moreover, both the IgL (Figure 6A, Blot: PY, 3 min exposure) and IgH (not shown) of the stimulating anti-CD3 ϵ mAb were also translocated specifically to DIMs upon TCR/CD3 binding, suggesting that the recruitment of TCR/CD3 to DIMs in stimulated thymocytes directly involved the engaged receptor complexes. The co-immunoprecipitation of TCR ζ chains with CD3 ϵ , under conditions in which DIMs were solubilized (Figure 6B), further suggests that the DIM-associated TCR/CD3 subunits remained in complexes. Interestingly, the TCR/CD3-containing DIMs seemed to display a mean density slightly higher than that of the bulk of DIMs (compare the distribution of TCR/CD3 subunits with that of Lck in the low-density fractions in Figure 5C). In this context, Cerny *et al.* (1996) have reported the sub-compartmentalization of kinase activity in the heavier subfractions of DIMs in Jurkat T cells. Collectively, our results strongly suggest that TCR/CD3 ligation promotes a selective association of engaged receptor complexes with DIMs in mouse thymocytes.

Fig. 4. Association of GPI-anchored proteins and GM1 in DIMs. (A) Co-isolation of GPI-anchored proteins and GM1. Surface-biotinylated proteins of mouse thymocytes (2×10^8) were separated into DIM and H fractions before immunoprecipitation with specific mAbs to different GPI-anchored proteins, or with a CTB-anti-CTB complex that binds to GM1. The immunoprecipitates were resolved on 5–15% SDS-PAGE and blotted using the streptavidin–HRP/ECL system to reveal the biotinylated proteins. The positions of Thy-1 and ThB are indicated. The region of the gel corresponding to the migration front was blotted by the CTB-anti-CTB complex to reveal GM1. (B) Electron micrographs showing Thy-1 and GM1 distribution in DIM vesicles. DIM vesicles were immunogold labeled and analyzed by electron microscopy. (a) Negative control for DIM vesicle labeling, see Materials and methods; (b) DIM vesicles stained with CTB-anti-CTB plus 20 nm gold-labeled protein A; (c) DIM vesicles stained with an anti-Thy-1 mAb plus 10 nm gold-labeled anti-rat Ig; (d) DIM vesicles stained with CTB-anti-CTB plus 20 nm gold-labeled protein A and then with anti-Thy-1 mAb plus 10 nm gold-labeled anti-rat Ig.

Table I. Distribution of Thy-1- and GM1-specific gold labeling on DIM vesicles

	% of Thy-1-positive vesicles	Mean No. of 10 nm gold beads per vesicle ^a	% of GM1-positive vesicles	Mean No. of 20 nm gold beads per vesicle ^a
Single labeling	88	11.5	84	5.3
Double labeling	78	12.5	62	3.3

^aCalculated over all labeled vesicles.

TCR engagement triggers an immediate accumulation of a series of prominent P_{tyr} substrates in DIMs

The earliest detectable signaling event that follows TCR engagement is a series of tyrosine phosphorylations including those mediated by the Src kinases, Lck and Fyn (Weiss and Littman, 1994). We have examined the presence of phosphorylated tyrosine-containing (P_{tyr}) proteins in DIMs before and after TCR stimulation. Figure 6A depicts the kinetics of tyrosine phosphorylation in thymocytes stimulated for various time periods by anti-CD3ε; at each time point, P_{tyr} proteins in either DIMs or the H fraction were analyzed by immunoblot with an anti-phosphotyrosine mAb. In non-stimulated cells, the major P_{tyr} proteins observed had a mol. wt of ~60 kDa in both DIMs (as a doublet) and H fractions. Immunoprecipitation studies identified Lck as being one of these proteins (data not shown). TCR cross-linking by anti-CD3ε mAb very rapidly induced a series of P_{tyr} proteins which partitioned differentially into DIMs and the H fraction, resulting in a specific

pattern for each of the two fractions. Indeed, TCR cross-linking triggered the induction of P_{tyr} proteins with mol. wts of 23, 36, 68, 70–80, 120 and 200 kDa and the enhanced expression of a P_{tyr} protein of 21 kDa in DIMs, versus the induction of P_{tyr} proteins with mol. wts of 72, 100 and 110 kDa in H fractions. The identity of DIM-associated P_{tyr} proteins (except that of 21 and 23 kDa proteins, see below) currently is under investigation. The p36 P_{tyr} protein that was found to bind to the SH2 domain of GRB-2 (our unpublished observation) is possibly the recently cloned LAT protein (Zhang *et al.*, 1998) (see Discussion), while the p68 P_{tyr} protein is probably ZAP-70 kinase (see below). All the DIM-associated P_{tyr} proteins seemed to follow identical induction kinetics: they were readily detected after 10 s, reached a maximum after 2 min and were down-regulated after 20 min of TCR stimulation. It is noteworthy that these kinetics coincided precisely with those of TCR/CD3 recruitment to DIMs, as revealed by the redistribution of CD3ε. On the other hand, the level of some of H fraction-associated inducible

Fig. 5. TCR engagement promotes association of TCR/CD3 complexes with DIMs. (A) Distribution of TCR ζ and ThB in the different fractions of the sucrose density gradient. Thymocytes (2.5×10^8) were incubated with 15 $\mu\text{g/ml}$ of 145-2C11 anti-CD3 ϵ mAb or an irrelevant RB6-8C5 mAb at 37°C for 5 min. PNS preparations were solubilized in Brij 58 and fractionated on sucrose density gradients. Equal volumes from 1/20 of each fraction were analyzed on immunoblot with anti-TCR ζ and anti-ThB mAbs, respectively. (B) Association of TCR ζ with DIMs in thymocytes from female H-Y transgenic mice stimulated by antigen. Thymocytes (5×10^7) from female H-Y transgenic mice were incubated with APCs from C57/BL6 female or male mice, respectively, as described in Materials and methods. The presence of TCR ζ in the DIM and half of the H fractions (concentrated as described in Materials and methods), was analyzed by immunoblotting with anti-TCR ζ mAb. (C) Association of TCR $\alpha\beta$, ζ and CD3 ϵ with DIM fractions. Thymocytes (2.5×10^8) were stimulated as in (A). Proteins in one-quarter of the DIM fraction were analyzed on immunoblot with the indicated antibodies. Lck was also detected in the same DIM fractions. In all experiments shown, SDS-PAGE was performed in non-reducing conditions to detect TCR $\alpha\beta$ heterodimers ($\alpha\beta$) and the 32 kDa TCR ζ homodimers (ζ_2).

Ptyr proteins (e.g. p72) peaked after 10 s and was then down-regulated, while that of others (e.g. p110) was maintained throughout the induction time course.

Upon TCR stimulation, the DIM-associated receptor complexes are highly enriched in the inducible phospho- ζ , contain most of the TCR/CD3-associated phospho-ZAP-70 and are found within larger, multiple tyrosine-phosphorylated substrate-containing protein complexes

We identified the 21 and 23 kDa Ptyr proteins as TCR ζ p21 and ζ p23 chains based on their immunoprecipitation by an anti-TCR ζ mAb (Figure 6B) under conditions in which DIMs were solubilized (Figure 6B). In accordance with published data (van Oers *et al.*, 1994; Wiest *et al.*, 1996), ζ p21 was found to be a constitutive Ptyr protein, whereas phospho- ζ p23 is induced by TCR stimulation. The phosphorylation of both ζ p21 and ζ p23 is mediated by Lck in mouse thymocytes (van Oers *et al.*, 1996). The phospho- ζ p21 is probably responsible for the constitutive

association of non-phosphorylated ZAP-70 with TCR/CD3 (Wiest *et al.*, 1996), while induction of ζ p23 is correlated with the phosphorylation and subsequent activation of TCR-associated ZAP-70 which provokes an effective TCR-signaling cascade (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995; Wiest *et al.*, 1996). We observed that TCR stimulation resulted in an increase of non-phospho- ζ p16 and phospho- ζ p21 as well as the appearance of phospho- ζ p23 chains associated with DIMs. Remarkably, ζ p23 chains were found to be highly enriched in the DIM-associated TCR/CD3 complexes as compared with those present in H fractions, based on the ratio between ζ p23 to either ζ p21 or ζ p16 isoforms (Figure 6A and B). The increase in ζ p21 and ζ p16 in DIMs was likely to be the consequence of TCR/CD3 recruitment, as we failed to observe significant modifications in the total amount of these ζ isoforms after TCR stimulation (data not shown). However, the DIM's ζ p23 could reflect either a recruitment of TCR/CD3-associated ζ p23 or, alternatively, a phosphorylation event occurring in DIMs.

Fig. 6. Accumulation of Ptk proteins in DIMs upon TCR stimulation. **(A)** Kinetic analysis of Ptk proteins upon TCR stimulation in DIM and H fractions. Thymocytes (2.5×10^8) were stimulated with 15 $\mu\text{g}/\text{ml}$ of 145-2C11 anti-CD3 ϵ mAb at 37°C for various periods of time, as indicated. The non-stimulation (0) point corresponded to a 5 min incubation at 37°C with the irrelevant RB6-8C5 mAb (15 $\mu\text{g}/\text{ml}$). Proteins in one-quarter of the DIM and 1/40 of H fractions were concentrated and resolved on 12% SDS-PAGE, followed by blotting with 4G10 anti-phosphotyrosine mAb. The arrowheads indicate the position of major TCR-induced Ptk proteins in DIMs, whereas the arrows indicate the position of those detected in H fractions. Longer exposures were performed to improve the analysis of the 20–40 kDa region, and the position of phospho- ζ p21, p23 and p36 Ptk proteins is indicated. The detection of IgL, due to the binding of the secondary HRP-conjugated antibody, is also shown. The blot subsequently was stripped and reprobed for the detection of CD3 ϵ and Lck. The latter was used as an internal control for protein loading. **(B)** Immunoprecipitation analysis by anti-TCR ζ mAb in DIM and H fractions from non-stimulated and TCR-stimulated thymocytes. Thymocytes (2.5×10^8) were stimulated for 5 min or not as in (A). DIM and H fractions were prepared and treated with 60 mM *N*-octylglucoside for 1 h at 4°C, before being subjected to immunoprecipitation by an anti-TCR ζ mAb coupled to Eupergit C1Z beads. Immunoprecipitation control was obtained by using Eupergit C1Z beads coupled to an irrelevant Ig. Immunoprecipitates were resolved on 7–17% SDS-PAGE and blotted with 4G10 anti-phosphotyrosine mAb. The position of phospho- ζ p21 and p23, phospho-ZAP-70, IgL and IgH is indicated. Note that Ig signals in the blot are not due to the binding of 4G10, but to that of the goat anti-mouse HRP-conjugated secondary antibody to the hamster anti-TCR ζ mAb used for immunoprecipitation. The arrowheads indicate the position of four unknown major TCR/CD3-associated Ptk proteins upon stimulation, with mol. wts of 28, 36, 40 and 120 kDa, respectively. The blot was then stripped and reprobed with anti-TCR ζ (this mAb only detects the non-phospho- ζ p16 in reducing conditions) and anti-ZAP-70. **(C)** Co-immunoprecipitation of TCR ζ with CD3 ϵ in DIM and H fractions of TCR-stimulated thymocytes. Thymocytes (2.5×10^8) were stimulated as in (B). DIM and H fractions were prepared and treated with 60 mM *N*-octylglucoside for 1 h at 4°C. CD3 ϵ bound to 145-2C11 mAb used for the stimulation was precipitated with protein A-coupled Sepharose. Immunoprecipitation control was obtained by using protein A-coupled Sepharose pre-saturated with hamster Igs. Immunoprecipitates were resolved on 12% SDS-PAGE and blotted with 4G10 anti-phosphotyrosine mAb. The blot was then stripped and reprobed with anti-TCR ζ to reveal the non-phospho- ζ p16.

The DIM-associated, ζ p23-rich TCR/CD3 complexes appeared directly to concern the engaged receptor population, as such an enrichment was observed similarly in TCR/CD3 complexes precipitated via the anti-CD3 ϵ mAb

added during cell stimulation (Figure 6C). In agreement with ζ p23 enrichment in TCR-stimulated thymocytes, the phosphorylated, and thus activated, ZAP-70 kinases were found dominantly associated with TCR/CD3 located inside

DIMs, despite the low content of TCR/CD3/ZAP-70 complexes in these membrane compartments as compared with H fraction (Figure 6B). Moreover, the DIM-associated TCR/CD3 complexes in TCR-stimulated thymocytes were characterized as being associated with multiple tyrosine-phosphorylated substrates (Figure 6B), that together probably constituted parts of higher order molecular signaling complexes (Germain, 1997). Taken together, these data strongly suggest that DIMs represent a membrane compartment in which engaged TCR/CD3 complexes are concentrated and activated to initiate the effective signaling cascades.

Sensitivity to cholesterol sequestration and isolation via GPI-anchored proteins of the TCR/CD3-containing DIMs

A fundamental feature of DIMs is their solubilization in the detergent to which they are normally resistant, upon a cholesterol sequestration (Brown and London, 1997; Schroeder *et al.*, 1998). Figure 7A shows that when cells were lysed in Brij 58 in the presence of 0.2% saponin, neither the non-activated nor the phosphorylation-activated TCR/CD3 chains were still detected in the light fractions of the density gradient from the non-stimulated or TCR-stimulated thymocytes, respectively. The same result was obtained when cholesterol was pre-depleted from the membrane by 0.2% saponin before adding Brij 58 to the cells, while saponin by itself did not solubilize TCR/CD3 (data not shown). These data demonstrate that the detergent-insoluble light membranes that contain TCR/CD3 from both non-stimulated and stimulated thymocytes represent the authentic DIMs.

We (our unpublished observations) and Bohuslav *et al.* (1993) have found that Triton X-100 (or NP-40) preserved the DIM structure less completely than does Brij 58 under classical solubilization conditions in mouse thymocytes and EL-4 thymoma cells, respectively. Field *et al.* (1995, 1997) recently have reported that the aggregation-dependent association of Fc ϵ RI with DIMs in RBL-2H3 cells can be analyzed properly by lowering the Triton X-100/cell ratio during cell lysis. Figure 7B shows that when thymocytes were solubilized in 0.2% Triton X-100, a better preservation of DIMs was obtained, as suggested by the substantial recovery of Lck in the detergent-insoluble, buoyant membrane fractions (which, nonetheless, is lower than in 1% Brij 58). These membranes are indeed DIMs as they become sensitive to the same concentration of Triton X-100 after cholesterol depletion and are devoid of TfR (not shown). By analyzing thymocyte DIMs under these solubilization conditions, we confirmed the two major points in our study: (i) TCR engagement promotes its recruitment to DIMs; and (ii) the DIM-recruited TCR is enriched in the phosphorylation-activated ζ p23 chain.

To investigate the association of TCR/CD3 with DIMs further, DIM vesicles isolated from a sucrose density gradient subsequently were immunoprecipitated by Thy-1 mAb. We reasoned that since only those DIM vesicles directly bound to Thy-1 mAb are isolated under our immunoprecipitation conditions (Figure 3), all the markers that can be co-immunoprecipitated by anti-Thy-1 mAb should be present in the same DIM vesicles as GPI-anchored proteins. Figure 7C shows that anti-Thy-1 mAb

specifically isolated DIM vesicles that contain TCR/CD3 chains, ZAP-70, Lck and GM1, from both non-stimulated and TCR-stimulated thymocytes (only the latter contain ζ p23 and phospho-ZAP-70), respectively. GM1 instead of Thy-1 was used to monitor the amounts of DIM vesicles in the immunoprecipitates because the co-migration of IgL with Thy-1 in the gel interferes with its proper detection on the blot. Importantly, a similar ratio was found for each marker between the content in the starting material and in the Thy-1 precipitates. These results indicate that Thy-1 mAb isolated all light membrane vesicles with the same efficiency regardless of the presence of TCR/CD3 or Lck, and support the conclusion that most of TCR/CD3, ZAP-70 and Lck are present in the same DIM vesicles as GPI-anchored proteins. Moreover, the results of these experiments fully confirmed the TCR-triggered recruitment of TCR/CD3 chains and ZAP-70 to DIMs, as well as the concomitant detection of ζ p23 and phospho-ZAP-70 in such membrane compartments. Interestingly, we found a low, but detectable level of non-phosphorylated ZAP-70 present in DIMs in non-stimulated thymocytes. This result is in contrast with that showing that anti-TCR ζ mAb could not co-precipitate ZAP-70 (via phospho- ζ p21) from the DIMs solubilized in *N*-octylglucoside, but did so from H fractions in the same cells (Figure 6B). Together, these results argue that the presence of TCR/CD3 in DIMs is not due to an *in vitro* contamination by the TCR/CD3 in the H fraction, and that the engagement of the receptor is required for the DIM association of TCR/CD3 complexes stably linked to ZAP-70, and thus more competent for activation.

TCR engagement triggers the activation of DIM-associated Lck

In addition to promoting P_{Tyr} substrate accumulation in DIMs, TCR engagement was found to trigger the activation of Lck in the same membrane domains. Figure 8 depicts an *in vitro* kinase assay in which the activity of Lck was measured by its phosphorylation of a ζ -derived ITAM-containing peptide as a substrate. Quantitation indicated a 2-fold increase in the total activity for DIM-associated Lck in mouse thymocytes upon TCR stimulation. This value probably underestimates the activation process, since TCR-triggered Lck activation is probably observed only in a subset of thymocytes (Wiest *et al.*, 1996). Moreover, such an activation might concern only a fraction of DIM-associated Lck in a given cell (see Discussion). However, in any case, these data suggest that Lck-mediated phosphorylation events occur in DIMs following TCR engagement, and further support a positive role for DIMs in TCR signaling.

Inhibition of Src family kinase activity by PP1 interferes with the TCR/CD3 recruitment to DIMs upon TCR stimulation

To investigate the relationship between TCR/CD3 recruitment to DIMs and the Src kinase-mediated phosphorylation *in vivo*, we evaluated the effect of PP1, a specific inhibitor of the Src family kinases (Hanke *et al.*, 1996) (Figure 9). Treatment of thymocytes with 10 μ M PP1 abolished TCR-triggered phosphorylation by Src family kinases, as shown by the failure to detect the phospho- ζ p23, as well as the other inducible P_{Tyr} substrates

Fig. 7. TCR/CD3-containing DIM vesicles are sensitive to cholesterol sequestration and can be isolated via GPI-anchored proteins. **(A)** Effect of saponin on Brij 58 solubility of TCR/CD3-containing DIMs. Thymocytes (2.5×10^8) were stimulated or not as in Figure 5A. PNS preparations were treated with either 1% Brij 58 or 1% Brij 58 plus 0.2% saponin at 4°C and fractionated on sucrose density gradients. Proteins in one-quarter of the DIM and 1/20 of the H fractions were concentrated and resolved on 12% SDS-PAGE. The TCR/CD3-containing DIMs were detected by blotting with anti-CD3ε mAb and anti-phosphotyrosine mAb. Lck staining was used as an internal control for DIM solubilization. **(B)** TCR/CD3-containing DIMs isolated from mouse thymocytes using 0.2% Triton X-100. Thymocytes (2.5×10^8) were stimulated or not as in (A). PNS preparations were treated with 0.2% Triton X-100 at 4°C and fractionated on sucrose density gradients. Proteins in one-quarter of the DIM and 1/20 of the H fractions were concentrated and resolved on 12% SDS-PAGE. The TCR/CD3-containing DIMs were detected as in (A). **(C)** Thymocytes (2.5×10^8) were stimulated or not as in (A). PNS preparations were treated with 1% Brij 58 and fractionated on sucrose density gradients. One-quarter of the DIM vesicles were either concentrated by ultracentrifugation or subjected to immunoprecipitation by anti-Thy-1 mAb-coupled Eupergit C1Z beads. Immunoprecipitation control was obtained by using Eupergit C1Z beads coupled with an irrelevant Ig. Immunoprecipitates were resolved on 7–17% SDS-PAGE and blotted with specific antibodies as indicated. 4G10 anti-phosphotyrosine mAb was used to probe phospho- ζ p21, phospho- ζ p23 and phospho-ZAP-70. The 70 kDa region of the blot was then stripped and reprobed with anti-ZAP-70. GM1, used as an internal control for the amounts of DIM vesicles collected, was revealed by biotinylated CTB/streptavidin-HRP.

(e.g. p36 Tyr protein) in DIMs or H fractions (not shown). Under such conditions, the recruitment of TCR/CD3 to DIMs upon anti-CD3ε mAb cross-linking was no longer observed, as indicated by the amount of ζ p21 and ζ p16 in DIMs. However, this recruitment still occurred to some extent in the presence 5 μ M PP1, though the TCR-mediated tyrosine phosphorylation was barely detected. Thus, these results indicate that recruitment of engaged TCR/CD3 to DIMs is dependent on the TCR-triggered phosphorylation mediated by Src family kinases, through a highly efficient mechanism.

Discussion

In this report, we have shown the recruitment of TCR/CD3 to DIMs, which is dependent on both receptor

engagement and the activity of Src family kinases. Upon TCR stimulation, the DIM-associated receptor complexes are highly enriched in hyperphosphorylated ζ p23 chains and contain most of the TCR/CD3-associated and phosphorylation-activated ZAP-70 kinases. Moreover, such TCR/CD3/ZAP-70 complexes seem to be found within higher order supra-molecular signaling complexes. These findings, together with the observations of the TCR-triggered, immediate accumulation of the inducible Tyr proteins as well as the activation of Lck in DIMs, suggest that TCR/CD3 recruitment to a specific membrane domain could represent an important, so far previously unrecognized, biochemical event in TCR-mediated signal transduction. Our data are thus in accordance with the proposition that DIMs participate in cellular functions via regulated concentration and

Fig. 8. Increase of DIM-associated Lck activity upon TCR engagement. Thymocytes (2.5×10^8) were stimulated or not as in Figure 5A. DIM fractions were prepared and the DIM-associated Lck was solubilized in 1% Brij 58 plus 0.2% saponin at 4°C. Following immunoprecipitation by Eupergit C1Z beads coupled with an anti-Lck mAb, the immune complex was analyzed in a kinase assay by using TCR ζ -derived ITAM peptide as a substrate, as described in Materials and methods. Immunoprecipitation control was obtained by using Eupergit C1Z beads coupled with an irrelevant Ig. Immunoprecipitates were resolved on 7–17% SDS-PAGE under non-reducing conditions, and ³²P-labeled ITAM-containing peptides were scanned by a Fuji imager. The quantitation indicated a 2-fold increase of DIM-associated Lck activity in TCR-stimulated thymocytes. The amounts of Lck in immunoprecipitates were checked by blotting with an anti-Lck mAb.

Fig. 9. Inhibition of Src family kinase activity by PP1 interferes with the recruitment of TCR/CD3 in DIMs upon TCR stimulation. Thymocytes (2.5×10^8) were pre-treated with 0, 5 or 10 μM PP1 for 10 min at 37°C before being submitted to TCR or mock stimulation as in Figure 5A. Phospho- ζ p21 and p23 chains and p36 P_{tyr} protein in (1/4 of) the DIM fraction were analyzed on immunoblot with 4G10 anti-phosphotyrosine mAb. The blot subsequently was stripped and reprobed for the detection of ζ p16 and Lck, respectively. The positions of phospho- ζ p21 and p23, p36 P_{tyr} protein and ζ p16 are indicated.

exclusion of specific membrane proteins (Harder and Simons, 1997; Simons and Ikonen, 1997).

The role of DIMs in TCR signaling has been investigated using mouse thymocytes. This cell system is of particular interest because it is suitable for the biochemical studies of DIMs that require a high number of cells and because DIM function in signal transduction can be analyzed in normal, non-transformed cells. Tumor cells are known to display modifications in their plasma membrane in terms of GSL and cholesterol contents (Dainiak, 1991), and shed surface gangliosides in substantial quantities (Bergelson, 1995). In addition, Koleske *et al.* (1995) found that cell transformation was correlated negatively with caveolae

expression in fibroblasts. It should be borne in mind, however, that thymocytes are composed of heterogeneous cell populations, and our functional analysis concerns only the more mature thymocyte subsets (Wiest *et al.*, 1996). We have used Brij 58 (Bohuslav *et al.*, 1993) to isolate optimally the low-density, detergent-resistant membrane complexes in mouse thymocytes that fulfilled all known structural features of DIMs. It is not known why Brij 58 preserves DIM structures more efficiently than does Triton X-100 in these cells. The sensitivity of DIMs to mild detergents presumably reflects the defined composition of lipids (sphingolipids, cholesterol, etc.) and proteins, as well as the molecular interactions within DIMs in a given cell type. Nonetheless, we found it possible to analyze thymocyte DIMs in Triton X-100 by lowering the detergent/cell ratio during cell lysis, similarly to the previous observations made for DIMs from RBL-2H3 cells (Field *et al.*, 1995, 1997). In addition, the sorting experiment with a mixture of Thy-1.1 and Thy-1.2 thymocytes has demonstrated that DIMs do not trap other proteins or membrane fragments during the (Brij 58) detergent extraction procedure. Similar findings arguing against detergent-induced artifacts were reported previously using a human/murine cell system (Bohuslav *et al.*, 1993) or DIM-containing liposomes (Schroeder *et al.*, 1998). Finally, EM analysis of DIMs isolated from mouse thymocytes indicates that in contrast to caveolae-containing cells (Schnitzer *et al.*, 1995), GPI-anchored proteins and GSLs are co-distributed within DIMs in mouse thymocytes.

Biophysical studies have strongly supported the existence of raft domains (Brown and London, 1997; Hooper, 1998), for instance by establishing a solid correlation between the presence of liquid-ordered phase and the detergent insoluble lipid domains in model membranes (Schroeder *et al.*, 1994, 1998; Ahmed *et al.*, 1998), and by revealing a cellular GSL content-dependent, Triton X-100-insensitive, transient confinement of Thy-1 and GM1 at the surface of fibroblasts (Sheets *et al.*, 1997). Moreover, EM analysis recently has enabled the direct *in situ* visualization of membrane domains that are enriched in raft domain markers. Following a rigorous fixation procedure, a prominent clustered distribution of GPI-anchored molecules, GSLs and cholesterol was observed in the plasma membrane (Danielsen and van Deurs, 1995; Fujimoto *et al.*, 1997; Stauffer and Meyer, 1997; Wu *et al.*, 1997). Several of these studies pointed out that both mild early fixation (Wu *et al.*, 1997) and probe binding (Smart *et al.*, 1996; Fujimoto *et al.*, 1997) could alter the native patched distribution of these molecules in the membrane, providing a possible explanation for the failure to detect these structures in previous investigations (Mayor and Maxfield, 1995).

In this study, we have shown for the first time that the TCR engagement promoted TCR/CD3 recruitment to DIMs in T lymphocytes. The TCR/CD3-containing light membranes, with a mean density slightly higher than that of the bulk of DIMs, were characterized unambiguously as authentic DIMs based on their GPI-anchored protein content and their detergent sensitivity upon cholesterol depletion. Furthermore, our data are consistent with recent studies using HPB-ALL T cells. Thus, Cerny *et al.* (1996) observed a low amount of CD3 present in the heavier subfraction of DIMs from resting cells lysed in Brij 58;

in addition, Marano *et al.* (1997) have described the aggregation-dependent TCR insolubility in Triton X-100, which was unaffected by the inhibition of actin or tubulin polymerization. It is indeed unlikely that TCR association with DIMs relates to the previously reported TCR ζ -actin cytoskeleton association. Thus, although the association of proteins with both DIMs and cytoskeleton results in their detergent insolubility, saponin is only known to promote the detergent solubilization of DIMs but not of cytoskeleton complexes through its cholesterol sequestration capability. Our conclusion is supported further by the work of Caplan and Baniyash (1996) showing that a unique phospho-isoform of TCR ζ (phospho- ζ p16), but not the phospho- ζ p21 nor the phospho- ζ p23, is associated with the detergent-insoluble actin cytoskeleton in mouse thymocytes and splenocytes either before or after activation, while in our study phospho- ζ p21 and phospho- ζ p23 were the only phospho-isoforms of TCR ζ shown to be associated with DIMs.

The quasi-instantaneous TCR/CD3 recruitment to DIMs along with the high enrichment of phospho- ζ p23 and the concentration of most of TCR/CD3-associated, phosphorylation-activated ZAP-70 in DIMs upon receptor engagement, led us to propose a role for DIMs in the effective initiation of TCR signaling cascades. Indeed, the induction of phospho- ζ p23 and phospho-ZAP-70 has been correlated with the activation of ZAP-70, that already forms a complex with TCR/CD3 in mouse thymocytes and T lymphocytes (Wiest *et al.*, 1996), and the initiation of the productive TCR signaling (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995; Wiest *et al.*, 1996). Our proposition is in agreement with the results of Kabouridis *et al.* (1997) showing the essential role of *S*-acylation of Lck, which is the key protein tyrosine kinase (PTK) in the activation of the TCR/CD3/ZAP-70 complex, in TCR-dependent signaling pathways (Kabouridis *et al.*, 1997). Thus, these authors have shown that neither the non-*S*-acylated cytosolic form nor a transmembrane chimera of Lck (even in the presence of a cytosolic activated form), in contrast to the wild-type molecule, was able to support TCR signaling in Jurkat T cells. Reconstitution of some early TCR-signaling events, but not those involved in the induction of CD69 and NFAT late signaling proteins, is achieved when transmembrane Lck and TCR are brought into close proximity artificially by antibody-mediated cross-linking. The fact the non-*S*-acylated cytosolic and transmembrane forms of Lck molecules were found to be excluded from DIMs leads to the suggestion that the Lck molecules enriched in such domains actively participate in TCR-mediated signal transduction, and are required for the achievement of full signaling pathways. In fact, the recruitment of the receptor complex observed in our study probably represents the natural way in which the engaged TCR/CD3/ZAP-70 meets the DIM-associated Lck.

The immediate accumulation of a specific group of prominent Tyr proteins in DIMs, induced by TCR engagement, also argues for the positive role of DIMs in TCR signaling. Particularly interesting among these proteins is a major substrate of PTK of 36 kDa, possibly corresponding to the 36 kDa Tyr protein described by Kabouridis *et al.* (1997), which was phosphorylated normally in the TCR-stimulated wild-type Jurkat cells, but did not undergo a sustained phosphorylation when the transmembrane Lck

chimera was cross-linked to the TCR. Recently, this p36 Tyr protein was shown to be DIM associated, and was identified as the LAT protein (T.Magee, personal communication). In our preliminary experiments, the TCR-induced, DIM-associated p36 Tyr protein in mouse thymocytes was found to interact with the SH2 domain of Grb-2 (data not shown), known to be a characteristic of LAT (Zhang *et al.*, 1998). Taken together, these observations, in the context of the central role of LAT as the ZAP-70 kinase substrate that links TCR to T-cell activation (Zhang *et al.*, 1998), reinforce the possibility that TCR engagement promotes the ZAP-70 kinase-dependent activation events in DIMs which couple TCR to downstream signaling cascades.

Higher order oligomerization of TCR/CD3 together with other membrane and cytosolic proteins has been proposed to be critical for sustained TCR signaling (Germain, 1997; Shaw and Dustin, 1997). Interestingly, our results showed that upon TCR stimulation, DIM-associated TCR/CD3 molecules were found within multiple tyrosine-phosphorylated substrate-containing protein complexes which may reflect the presence of a higher order supra-molecular organization. In addition, the TCR/CD3-containing DIMs are slightly heavier than the bulk of DIMs, possibly due to an increase in their protein/lipid ratio caused by the presence of a high-order protein association in these DIMs. The recruitment of TCR/CD3 could be the direct consequence of such an oligomerization induced by TCR ligation. For instance, TCR/CD3 clusters could strengthen their association with DIMs by means of multiple cooperative weak interactions (Harder and Simons, 1997). Conversely, the assembly of TCR-containing protein aggregates could be facilitated in DIMs by the confined protein-protein interactions. In fact, it is possible that the dynamic clustering and the DIM recruitment of TCR/CD3 complexes are the two aspects of the same strategy utilized by T cells to ensure a stabilized higher order molecular organization of signaling proteins that concentrate second messenger molecules and adaptor proteins and restrict negative regulators such as CD45 PTP, leading to the effective activation of TCR-signaling cascades (Rodgers and Rose, 1996; Germain, 1997; Shaw and Dustin, 1997).

The blockade of the engaged TCR/CD3 recruitment to DIMs by the inhibition of Src family kinase activity suggests that the Lck- (and Fyn)-mediated phosphorylation promotes or alternatively stabilizes TCR/CD3 association with DIMs upon receptor engagement. The former hypothesis is compatible with the possibility that an initial phosphorylation of TCR/CD3/ZAP-70 precedes the receptor complex recruitment to DIMs, while the second is compatible with the DIM-dependent phosphorylation of receptor complexes. Whichever the case, we would like to propose that DIMs participate in the effective TCR signaling by contributing to a reciprocal activation between Lck kinases and the phosphorylated TCR/CD3/ZAP-70 receptor complexes, based on our finding that TCR engagement triggered the activation of Lck in DIMs. The phospho-ZAP-70 and phospho- ζ were reported to interact with Lck SH2 (Thome *et al.*, 1995; Straus *et al.*, 1996) and SH2.3 (August and Dupont, 1996) domains, respectively. These interactions, suggested to be essential for the role of Lck in TCR signaling (Weiss and Littman, 1994; Straus

et al., 1996), are likely to be potentiated strongly by the elevated local concentration of Lck and by the confined lateral movements of the proteins inside DIMs. The binding of DIM-associated Lck to the phosphorylated fraction of ZAP-70 and ζ present in the recruited receptor complexes (which do not need to be fully phosphorylated) would heighten its activity by interfering with the intramolecular negative regulatory interaction between the SH2 domain and the C-terminal Tyr505 (Thomas and Brugge, 1997), probably phosphorylated to a large extent on the DIM-associated Lck in non-stimulated T cells (Rodgers and Rose, 1996). This could, in turn, result in further phosphorylations in TCR ITAMs and ZAP-70 as well as in other substrates, thus establishing a processive signaling mechanism. Such an activation process eventually will be down-regulated, for instance by signaling inhibitors, which could involve the low amount of CD45 present in DIMs. Similar mechanisms may exist for the receptor-mediated activation of Src family kinases in DIMs in other cells (Field *et al.*, 1995; Mastick and Saltiel, 1997).

Our study is reminiscent of the recent work on the compartmentalized activation of Fc ϵ RI within DIMs in RBL-2H3 cells (Field *et al.*, 1997; Stauffer and Meyer, 1997). Fc ϵ RI, structurally homologous to the TCR, is a multisubunit receptor composed of an IgE recognition/binding chain (α) and two ITAM-containing signal-transducing subunits (β and γ) (Metzger, 1992). The γ chain is related to TCR ζ and can partially replace this chain in the assembly of functional TCR complex (Shaw and Dustin, 1997). Field *et al.* (1997) observed that aggregation provoked the recruitment of Fc ϵ RI to DIMs, followed by the tyrosine phosphorylation of β and γ chains. More recently, Stauffer and Meyer (1997) have successfully monitored in living cells an antigen-stimulated, transient recruitment and phosphorylation of Fc ϵ RI and Syk kinase in GSL-enriched membrane domains, confirming the biochemical study. In the study by Field *et al.* (1997), the DIM recruitment of Fc ϵ RI appeared to be phosphorylation independent, consistent with the kinetic analysis. The reason for the observed difference in the phosphorylation dependency between TCR and Fc ϵ RI recruitment to DIMs is not clear. One explanation would be that the strong cross-linking via the biotin–streptavidin system and the use of limiting amounts of Triton X-100 detergent in Fc ϵ RI experiments may allow the detection of an (additional) phosphorylation-independent interaction of aggregated receptors with DIMs. In any case, the present study on TCR, along with those of Fc ϵ RI, supports the involvement of DIMs in the signal transduction of multichain immune receptors that utilize Src family kinases during their initial signaling step (Field *et al.*, 1997).

Despite extensive investigation, the precise role of GPI-anchored proteins in T-cell signaling is still poorly understood. Recent studies using mutant mice or cell lines suggest their involvement in regulating TCR-mediated signal transduction (Hueber *et al.*, 1997; Romagnoli and Bron, 1997; Stanford *et al.*, 1997). It would be of interest to examine whether GPI-anchored proteins are able to modulate TCR–DIM interactions. Indeed, several studies have shown that the co-cross-linking of GPI-anchored proteins with TCR/CD3 results in a strong synergized TCR-dependent signaling (Nakashima *et al.*, 1991; Cerny *et al.*, 1996). This finding fits with our model of DIM

involvement in TCR signaling, since such a co-cross-linking process would very probably facilitate the recruitment of TCR/CD3 to DIMs.

In summary, the results presented herein suggest a compartmentalized activation of TCR signaling in specialized membrane domains. Further investigations, for instance through *in vivo* immunofluorescent visualization (Stauffer and Meyer, 1997), will be required to complement the biochemical analysis as performed in this study. Nevertheless, it seems strongly warranted to integrate TCR–DIM interactions among other membrane-proximal events occurring during TCR signaling, such as the differential stimulation by agonist/antagonist peptides (Sloan-Lancaster *et al.*, 1994; Madrenas *et al.*, 1995), the oligomerization of TCR with co-receptors and accessory molecules (Germain, 1997; Shaw and Dustin, 1997), the serial TCR triggering (Viola and Lanzavecchia, 1996) and the association of TCR with the cytoskeleton (Rozdzial *et al.*, 1995; Caplan and Baniyash, 1996).

Materials and methods

Reagents

Organic solvents were from Merck, protein A-coupled Sepharose beads from Pharmacia, protease and phosphatase inhibitors, CTB and saponin from Sigma, sulfosuccinimidyl-6-biotinamido hexanoate (Sulfo-NHS-LC-Biotin) and streptavidin-coupled agarose beads from Pierce, enhanced chemiluminescence (ECL) reagents from Amersham, Brij 58 and Eupergit C1Z beads from Fluka, *N*-octylglucoside from Boehringer Mannheim and PPI from Calbiochem.

Mice and antibodies

(C57BL/6 $\times$ CBA) F₁ mice (4–6 weeks old), used in most of the experiments, were bred in our animal facility. C57BL/6 (Thy-1.2) and C57BL/6PL (Thy-1.1) mice were purchased from Jackson Laboratory. H-2^b H-Y TCR transgenic mice were provided by H.von Boehmer and bred in our animal facility. 145-2C11 (anti-CD3 ϵ) and M1/69 (anti-HSA) hybridomas were obtained from the American Type Culture Collection, and the corresponding mAbs were produced in our laboratory; the other antibodies used were as follows: 4G10 (anti-phosphotyrosine) and anti-mouse Lck (3A5) mAbs from Upstate Biotechnology Inc.; anti-Lck (2102) and anti-Fyn (fyn3) rabbit IgG from Santa Cruz Biotechnology; RB6-8C5 mAb (anti-Ly6G, a myeloid differentiation antigen) from Pharmingen; H194-92 anti-Thy-1, H129-93 anti-Thy-1.2, H129-121 anti-TfR and 49-h4.3 anti-ThB (Hueber *et al.*, 1994) rat mAbs produced in our laboratory; Y-L1/2 (anti-tubulin β) mAb provided by C.Faivre-Sarrailh (IBDM, Marseille); OX-7 anti-Thy-1.1 mAb provided by R.Morris (Guy's Hospital, London); H28 (anti-TCR $\alpha\beta$) and H146-968 (anti-TCR ζ) mAbs provided by B.Malissen (CIML); anti-ZAP-70 rabbit antiserum provided by O.Acuto (Institut Pasteur, Paris); anti-rab5 rabbit antiserum provided by P.Chavrier (CIML) and anti-CTB rabbit antiserum provided by P.Boquet (Institut Pasteur, Paris). Horseradish peroxidase (HRP)-coupled antibodies and streptavidin, as well as rabbit anti-rat IgG were from Jackson Immunoresearch Laboratories.

DIM isolation

Fresh thymocytes ($2\text{--}2.5\times 10^8$) were sonicated gently (five 5 s bursts, 5 W; Vibracell, Bioblock Scientific) in 1 ml of ice-cold buffer A (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA) supplemented with a mixture of protease inhibitors (leupepin 1 $\mu\text{g/ml}$, pepstatin 1 $\mu\text{g/ml}$, chymostatin 2 $\mu\text{g/ml}$ and $\alpha 2$ macroglobulin 5 $\mu\text{g/ml}$). After centrifugation at 800 g at 4°C for 10 min, the post-nuclear supernatant (PNS) was incubated with Brij 58 at a final concentration of 1%, for 1 h at 4°C. The lysate was then adjusted to 1.33 M sucrose by the addition of 2 ml of 2 M sucrose prepared in buffer A, placed at the bottom of an ultracentrifuge tube and a step sucrose gradient (0.2–0.9 M with 0.1 M steps, 1 ml each) in buffer A was placed on top (Garcia *et al.*, 1993). It was centrifuged at 38 000 r.p.m. for 15 h in an SW41 rotor (Beckman Instruments Inc.) at 4°C. One ml fractions were harvested from the top. DIMs were recovered mainly from the low-density fractions 2, 3, 4 and 5. When not specified, DIM fraction is pooled fractions 2–5 and

H fraction is pooled fractions 8 and 9. Proteins were concentrated as described (Wessel and Flugge, 1983). For experiments in which the concentrated DIM and H fraction samples were assayed, gels normally were loaded with one-quarter of the total DIM fraction proteins, but with less H fraction proteins (the amount is specified in the figure legends) to avoid abnormal protein migration. When P_{tyr} protein analysis was performed, buffer A was supplemented with 1 mM NaVO₄, 10 mM NaP-P and 10 mM NaF.

Surface biotinylation

Thymocytes (2×10^8) were labeled for 15 min at 20°C with 500 µg/ml of Sulfo-NHS-LC-Biotin (freshly dissolved in dimethylsulfoxide) in 10 ml of biotinylation buffer (10 mM Na-borate, pH 8.8, 150 mM NaCl). The reaction was stopped by addition of 10 mM NH₄Cl and, finally, cells were washed in 50 ml of buffer A.

Glycolipid analysis

After ultracentrifugation (at 100 000 g, 2 h, 4°C) for maximal sucrose elimination, DIM fractions were subjected twice to lipid extraction with chloroform:methanol:H₂O (4:8:3 by vol.) as previously described (Dubois *et al.*, 1990). Gangliosides were separated from neutral GSLs on a 2.5 cm silica acid column (Unisil, 100–200 mesh) as described (Dubois *et al.*, 1984). Gangliosides were purified further on Sephadex LH20 in chloroform:methanol (2:1). TLC analysis was performed on glass or aluminium-backed HPTLC silica gel 60 plates (Merck). Ganglioside chromatography was performed in chloroform:methanol:0.25% KCl in H₂O (5:4:1) then visualized by resorcinol reagent. In some experiments, the CTB was used for GM1 ganglioside detection using experimental conditions described for glycolipid immunostaining (Dubois *et al.*, 1990). The GM1-bound biotinylated CTB was revealed by the streptavidin–HRP/ECL system. Neutral GSL chromatography was performed in chloroform:methanol:H₂O (65:25:4) and visualized by orcinol reagent. GSLs from total thymocyte membranes were extracted from the pellet obtained by ultracentrifugation of the PNS (100 000 g, 2 h, 4°C).

Transmission electron microscopy

All the following steps were performed at 4°C. DIM vesicles, concentrated by ultracentrifugation (100 000 g, 2 h) after dilution in buffer A, were deposited on glow-discharged carbon-coated grids (Baltec) for 3 min, and further saturated for 15 min with phosphate-buffered saline (PBS) containing 0.1% bovine serum albumin (BSA) (buffer B). For Thy-1 immunolabeling, samples were incubated for 15 min with the H194-92 anti-Thy-1 mAb diluted to 15 µg/ml in buffer B, washed with PBS and incubated for 15 min with 10 nm colloidal gold-labeled goat anti-rat IgG antibody (Biocell Research Laboratories) diluted to 1:20 in buffer B. For GM1 labeling, DIM fractions were incubated with CTB (15 µg/ml) in buffer A for 30 min at 4°C. After ultracentrifugation, washed DIM vesicles were deposited on treated grids, saturated with buffer B and incubated for 15 min with a rabbit anti-CTB antiserum (1:50). Samples were then incubated for 15 min with 20 nm colloidal gold-labeled protein A (Biocell Research Laboratories) diluted to 1:20 in buffer B. For double labeling experiments, GM1-labeled vesicles were stained further for Thy-1 antigen as described above. In the negative control experiments for double labeling, CTB was omitted in the first step and Thy-1 mAb was replaced by the RB6-8C5 irrelevant mAb. For EM visualization, samples were stained for 1 min with 1% uranyl acetate in distilled water, and observed on a Zeiss EM 912 microscope (Oberkochen).

In vitro thymocyte stimulation

Thymocytes (2.5×10^8) were incubated with 15 µg/ml of anti-CD3ε mAb 145-2C11 or with an irrelevant mAb (RB6-8C5) in complete medium at 37°C for different periods of time. Cells were then centrifuged at 10 000 g for 8 s. One ml of cold buffer A was then added immediately to cell pellets, followed by sonication as described above. For antigen-specific stimulation, thymocytes from *H-2^b*, H-Y TCR transgenic female mice (2×10^6 cells per well) were incubated for 1 h at 37°C with adherent APCs prepared from spleens of male or female *H-2^b* mice as previously described (Swat *et al.*, 1991). Briefly, freshly isolated splenic cell suspensions in complete RPMI medium were incubated for 2 h (at 37°C, 5% CO₂) in Petri dishes, followed by washing out of non-adherent cells. Adherent APCs were recovered with trypsin-EDTA buffer (Gibco-BRL), seeded at 2×10^6 cells per well and incubated overnight at 37°C.

Immunoprecipitation and Western blotting analysis

DIM and H fractions of mouse thymocytes were pre-incubated with 100 µl of pansorbin (Calbiochem) for 30 min at 4°C. The pansorbin-

absorbed fractions were immunoprecipitated by adding specific antibodies together with 30 µl of protein A-coupled Sepharose beads and 10 µg/ml of rabbit anti-rat IgG, for 2 h at 4°C. In some experiments, antibodies that are coupled directly to Eupergit C1Z beads (Grassel *et al.*, 1989) were used. For GM1 immunoprecipitation, 10 µg/ml of CTB together with a rabbit anti-CTB antiserum was used. For immunoprecipitation of TCR molecules, DIM and H fractions were treated first with 60 mM *N*-octylglucoside for 1 h at 4°C. TCRζ was immunoprecipitated by H146-968 mAb (Smith *et al.*, 1997) directly coupled to Eupergit C1Z beads. CD3ε in TCR-stimulated thymocytes was immunoprecipitated by adding 10 µl of protein A-coupled Sepharose beads which collected the 145-2C11 mAbs used for TCR stimulation. Immunoprecipitates were washed three times with buffer A containing either 0.5% Brij 58 or 60 mM *N*-octylglucoside and dissolved in SDS–PAGE sample buffer. Following SDS–PAGE, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore). Blots were then incubated with HRP–streptavidin or with specific antibodies and then the appropriate HRP-conjugated secondary antibodies. Protein detection was performed by ECL following the manufacturer's instructions. PVDF membranes to be reprobed were stripped of bound antibody using the Amersham ECL protocol.

Lck enzymatic activity assay

The PTK activity of Lck was determined by assaying the phosphorylation of a peptide derived from a cytoplasmic region of TCRζ that contains the third ITAM, as previously described (Hueber *et al.*, 1997). Briefly, DIM-associated Lck was solubilized in 1% Brij 58 plus 0.2% saponin at 4°C before immunoprecipitation by an anti-Lck mAb directly coupled to Eupergit C1Z beads. Anti-Lck immunoprecipitates were washed twice with 1 ml of kinase buffer (HEPES 20 mM, pH 7.5, 100 mM NaCl, 5 mM MgCl₂, 5 mM MnCl₂), and then incubated for 15 min at 25°C in 50 µl of kinase buffer with 10 µCi [γ -³²P]ATP, 2 µg of the TCRζ-derived ITAM peptide and 1 µM cold ATP. After addition of 15 µl of 5× sample buffer, the samples were boiled and subjected to 7–17% gradient SDS–PAGE, under non-reducing conditions. Gels were transferred to PVDF membranes and scanned on a bio-imaging analyzer (Bas1000, Fuji).

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Data from our recent experiments show that disruption of DIM integrity *in vivo* upon membrane cholesterol depletion by methyl- β -cyclodextrin results in defective phosphorylation-dependent TCR activation, consistent with a recent report by Xavier *et al.* [R.Xavier, T.Brenan, Q.Li, C.McCormack and B.Seed (1998) Membrane compartmentation is required for efficient T cell activation. *Immunity*, **8**, 723–732]; they also confirm that the DIM-associated, p36 P_{tyr} protein in TCR-stimulated mouse thymocytes is LAT.