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CD4 segregates into specific detergent-resistant T-cell membrane microdomains

Key words:
CD4; detergent-insoluble membranes; glycolipids; glycosylphosphatidylinositol-anchored proteins; Src-like tyrosine kinases

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Abstract: In T cells, glycolipids, glycoproteins attached to the membrane via a glycosylphosphatidylinositol (GPI) anchor, and Src-like tyrosine kinases are highly enriched in a membrane fraction resistant to solubilization by nonionic detergents. We have investigated the distribution of CD4 in T-cell membranes and found that approximately 10% of the CD4 co-receptor is associated with detergent-insoluble membrane microdomains, whilst the remaining 90% is in soluble membranes. Moreover, approximately 60% of the "insoluble CD4" is present in membrane microdomains containing GPI-anchored proteins and high glycolipid-dependent kinase activity, whereas the remaining 40% displays no association with GPI-anchored proteins and lacks glycolipid-associated kinase activity. These results indicate that CD4 segregates at least into three different membrane microenvironments: 1) soluble membranes; 2) insoluble membrane microdomains containing GPI-anchored proteins; and 3) insoluble membrane microdomains devoid of GPI-anchored proteins. The level of CD4 in insoluble membranes was not modified upon triggering activation by T-cell receptor-crosslinking but detectable amounts of CD3 subunits were recruited into these specialized membranes under those conditions. The physical separation of CD4 into different membrane microenvironments raises the possibility of that some of the multiple functions of CD4 might segregate into distinct types of lipid microenvironment. The fact that components of T-cell receptor/CD3 complex were recruited into insoluble membranes upon stimulation is consistent with the CD4 present in this membrane fraction might participate in T-cell receptor-triggered activation events.

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Glycosylphosphatidylinositol (GPI)-anchored surface proteins, Src-family protein tyrosine kinases and some of their intracellular substrates co-immunoprecipitate in glycolipid-enriched membrane microdomains (1, 2). The resistance of such microdomains to nonionic detergent extraction highlights the cohesiveness of the acyl chains of GPI-anchored glycoproteins, glycolipids (3), and other lipid-modified proteins such as tyrosine kinases of the Src family (2) and heterotrimeric G protein α subunits (4), and suggests a biological relevance for this type of confinement (5). Crosslinking of surface

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GPI-anchored proteins activates signaling pathways leading to Lck activation and T-cell proliferation (6–9). Membrane microdomains containing GPI-anchored proteins might be involved in this process by grouping together GPI-anchored proteins and Src-like tyrosine kinases (5, 10). In T lymphocytes, the detergent-insoluble membrane fraction is devoid of most transmembrane proteins but contains significant amounts of the CD4 and CD8 co-receptors (11, 12). In addition to the direct iodoacetamide-sensitive association between the CD4 cytoplasmic tail and the N-terminal domain of Lck mediated by cysteine residues (13), the CD4 present in insoluble membranes is associated with Lck in an indirect way mediated by glycolipids (11). The biological significance of this glycolipid-mediated alternative mode of association of CD4 with Lck remains unknown, but it might contribute to novel aspects of the CD4 molecule (14).

Detergent-insoluble membrane fractions can be isolated by biochemical means based on either their large size or low density by gel filtration chromatography (2) or centrifugation to equilibrium (15), respectively. Recent evidence suggests the existence of heterogeneity within the detergent-resistant membrane fraction obtained from a variety of sources (16–19). Here, we described three types of insoluble membrane microdomains in T cells: the first one containing CD4, GPI-anchored proteins, and high glycolipid-associated kinase activity; the second one containing CD4 and lacking GPI-anchored proteins and glycolipid-associated kinases; and the third type with GPI-anchored proteins, high glycolipid-associated kinase activity but without CD4. The distribution of CD4 in different microdomains might indicate a functional segregation of CD4 into distinct specialized membrane microenvironments. Our results showing the specific recruitment of T-cell receptor (TCR)/CD3 components in insoluble membranes after activation is consistent with that the CD4 present in this membrane fraction might participate in TCR-triggered activation events.

Material and methods

Material

Mouse monoclonal antibodies MEM-43 (anti-CD59, IgG2a), MEM-43/5 (anti-CD59, IgG2a), MEM-57 (anti-CD3-ε, IgG2a), MEM-92 (anti-CD3-ε, IgM); MEM-118 (anti-CD55, IgM), and MEM-115 (anti-CD4, IgG2a), were from the Institute of Molecular Genetics, Prague. MEM-43 mAb was used for immunoprecipitation and preclearing experiments, whereas MEM-43/5 mAb was used for immunoblot analysis. Leu 3A (anti-CD4, IgG1) was obtained from Becton Dickinson (Mountain View, CA, USA). Rabbit antisera to Lck was a generous gift from Dr. A. Veillette (McGill University, Montreal, Canada) (20). Antisera to CD3-ζ and to ZAP-70 were kind gifts from Dr. B. Alarcón (Centro de Biología Molecular, Madrid). Triton X-100, protein G-Sepharose, Sepharose 4B, and peroxidase-coupled cholera toxin B subunit were from Sigma (St. Louis, MO, USA). Anti-phosphotyrosine PY20 antibodies covalently coupled to peroxidase were from Transduction Laboratories (Lexington, KY, USA). Peroxidase-conjugated antibodies were from Pierce (Rockford, IL, USA). The HPB-ALL T-cell line was originally obtained from the laboratory of Dr. J. L. Strominger (Harvard University, Cambridge, MA, USA).

Detergent extraction procedures, gel chromatography and centrifugation to equilibrium

The procedures used to isolate detergent-insoluble complexes were essentially those described either by Brown and Rose (15) or by Cinek and Horejsi (2). $2-10\times10^7$/ml cells grown in RPMI medium supplemented with 10% of fetal calf serum were lysed for 30 min in 25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 5 mM iodoacetamide and 1% Triton X-100 at 4°C, and the lysate homogenized by passing the sample through a 22-gauge needle. To isolate detergent-resistant membranes by centrifugation to equilibrium (15), 2 ml of lysate were brought to 40% sucrose in a final volume of 4 ml and placed at the bottom of an 8-ml 5–30% linear sucrose gradient. Gradients were centrifuged for 18 h at 39,000 rpm at 4°C in a Beckman SW41 rotor. Fractions of 1 ml were harvested from the bottom of the tube and aliquots were subjected to immunoblot analysis. To isolate detergent-insoluble membranes by gel filtration chromatography (2), cell lysates were centrifuged at 14,000 rpm in a microfuge, and the postnuclear supernatant was fractionated in a 3-ml Sepharose 4B column, previously equilibrated in lysis buffer, at 4°C. Fractions of 0.3 ml were collected and analyzed.

Immunoblot analysis

Samples were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions and transferred to Immobilon-P membranes (Millipore, Bedford, MA, USA). After blocking with 5% (w/v) nonfat dry milk, 0.05% (v/v) Tween-20 in phosphate-buffered saline, membranes were incubated with the primary antibody for 4 h. After several washings, the membranes were incubated for 1 h with the appropriate goat secondary antibody coupled to horseradish peroxidase, washed extensively, and developed using an ECL Western blotting kit (Amersham, Buckinghamshire, UK). Quantifications were done using a 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA, USA).

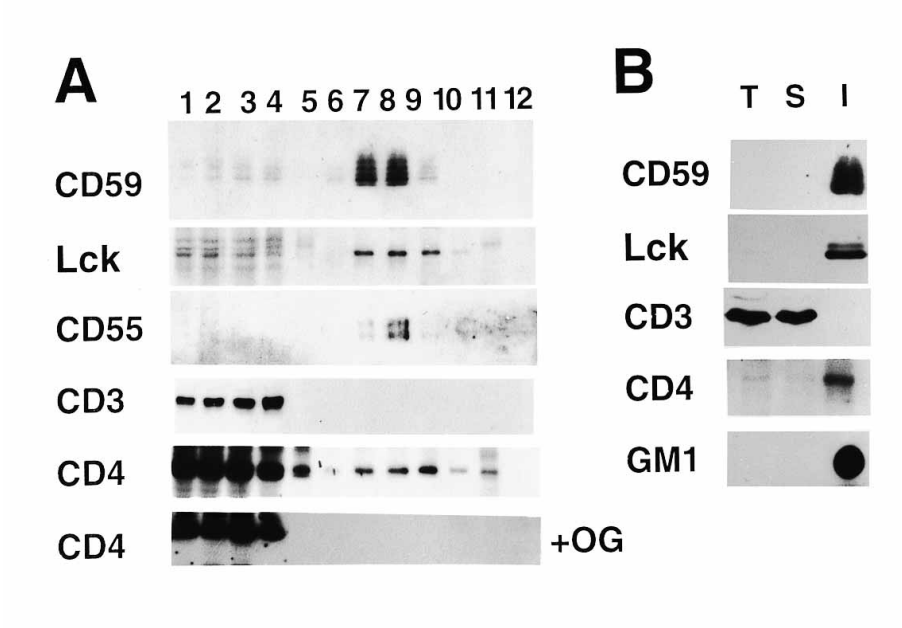

Fig. 1. **Isolation of detergent-insoluble membranes from HPB-ALL T-cells by centrifugation to equilibrium in sucrose density gradients.** HPB-ALL cells were extracted at 4°C with 1% Triton X-100, or with 1% Triton X-100 plus 60 mM octyl-glucoside (+OG). The extracts were centrifuged to equilibrium in sucrose density gradients, and fractionated from the bottom of the tube. **(A) Aliquots from each fraction were subjected to immunoblot analysis with antibodies to either CD59, CD55, CD4, CD3-ε or Lck.** Fractions 1–4 are the 40% sucrose layer and contain the bulk of cellular membranes and cytosolic proteins, while fractions 5–12 are the 5–30% sucrose layer and contain detergent-resistant membranes (15). **(B) Fractions 1–4 and 5–12 were pooled separately and named soluble (S) and insoluble (I) fractions, respectively.** Equal amounts of protein from the initial lysate (T) or from the S and I fractions were subjected to immunoblot analysis with anti-CD59, anti-CD4, anti-CD3-ε or anti-Lck antibodies, or to dot blot analysis using horseradish peroxidase-coupled to cholera toxin B subunit to detect the ganglioside GM1.

Lysate preclearing and *in vitro* kinase assay

Quantitative preclearing of samples was performed for 1 h on ice by incubating 0.5 ml of the fractionated lysates with 300 µl of Pansorbin coated with the indicated mAb. After centrifugation at 12,000 $\times g$ for 3 min at 4°C, the precleared supernatants were used for further experiments. For *in vitro* kinase assays, samples were immunoprecipitated using a modification of the solid phase immunoisolation technique (21). Briefly, wells of 96-well microtitration plates (Flow ICN, Meckenheim, Germany) were first coated with anti-mouse Ig and then with the indicated mouse mAb. After incubation with the cell lysates, the complexes immunoadsorbed in the wells were used for an *in vitro* kinase assay (22) performed using a reaction mixture containing 25 mM Hepes, pH 7.2, 5 mM $MnCl_2$, 0.1% Nonidet-40 and 0.1 µCi (γ-^{32}P)-ATP in a final volume of 50 µl. After 25 min of incubation at room temperature, wells were washed, and the material in the wells solubilized with sample buffer and analyzed by autoradiography after SDS-polyacrylamide gel electrophoresis.

Results

CD4 is enriched in detergent-resistant membranes

As an initial approach to characterize the detergent-resistant membrane fraction of HPB-ALL T cells, we used centrifugation to equilibrium in sucrose density gradients (15) of Triton X-100 cell extracts. Using this protocol, the low-density detergent-resistant membranes (fractions 5–12) are separated from the Triton X-100-solubilized material and cytosolic soluble proteins (fractions 1–4), and from the nuclei and cytoskeleton that pellet under these conditions. After centrifugation, aliquots from the different fractions were subjected to immunoblot analysis with antibodies to the GPI-anchored CD59 and CD55 antigens, to the CD4 molecule or to the Lck tyrosine kinase (Fig. 1A). Fig. 1A shows that large amounts of CD59, CD55, and Lck were detected in the low density fractions containing buoyant membranes, whereas CD3-ε and CD3-ζ were completely excluded from these fractions (not shown). Interestingly, the transmembrane co-receptor CD4 was also present in the insol-

Quantitative analysis of the fractions obtained by centrifugation to equilibrium *Table 1*

Fraction	%Protein	%CD4	%CD3-ε	%CD3-ζ	%ZAP-70	%Lck	%CD59	%GM1
S	99.4	90	>99.9	>99.9	>99.9	30	10	<0.1
I	0.6	10	<0.1	<0.1	<0.1	70	90	>99.9
Fold-enrichment (I/S)		~17	<0.2	<0.2	<0.2	~365	~1400	$>10^5$

Blots containing aliquots from fractions S and I were quantitated after Coomassie Blue staining (total protein), immunoblot analysis (CD4, CD3-ε. CD3-ζ, ZAP-70, Lck, CD59), or dot blot analysis with horseradish peroxidase-coupled to cholera toxin B subunit (GM1).

uble membrane fractions, although at levels lower than those of CD59, CD55 or Lck. In the presence of octyl-glucoside, a mild detergent known to disrupt glycolipid-enriched membranes (15), all of the CD4 appeared in the fractions contanining soluble proteins indicating that glycolipids are essential for CD4 insolubility. To demonstrate that these different types of proteins were indeed enriched in the insoluble membrane fraction, we compared the levels of CD59, Lck and CD4 using equal amounts of protein from the initial lysate (T) and from separated pools of fractions 1–4 (S) and fractions 5–12 (I). A remarkable enrichment in CD59, Lck, and CD4 but not in CD3-ε was clearly evident in the fraction containing insoluble membranes as shown in Fig. 1B. This fraction was also highly enriched in the ganglioside GM1 as demonstrated by dot-blot analysis with horseradish peroxidase coupled to cholera toxin B subunit. Table 1 shows a quantitation of the immunoblots shown in Fig. 1A together with the analysis of similar blots for the non-Src-like tyrosine kinase ZAP-70 and for CD3-ζ. Densitometric analysis of blots stained with Coomassie Blue revealed that the insoluble membrane fractions account for approximately 0.6% of the total protein. Quantitative analysis of the blots performed with anti-CD4 antibodies indicated that the insoluble membrane fractions contain approximately 10% of the CD4 molecules. This gives a 17-fold enrichment for CD4 in the insoluble membrane fractions relative to the soluble fractions. In contrast, other integral membrane proteins such as the ε or the ζ subunits of CD3 were totally excluded from the insoluble membrane fractions. A higher enrichment in the insoluble membrane fractions than that of CD4 was obtained for the lipid-anchored membrane proteins CD59, CD55 and Lck. Finally, ZAP-70 was totally excluded from the insoluble membrane fraction.

We then compared the insoluble membranes obtained by centrifugation to equilibrium with those isolated using molecular filtration chromatography. Triton X-100 extracts from HPB-ALL cells were centrifuged to remove nuclei, cytoskeleton and denatured protein aggregates, and the postnuclear supernatant was subjected to fractionation on a Sepharose 4B column. Following this established protocol (2), insoluble membranes appear in the void volume fractions (fractions 4–5) whilst the soluble and the solubilized material appear in the included fractions (fractions 6–10). In agreement with the results shown in Fig. 1, CD59, CD55 and Lck (not shown) were mostly present in the void volume fractions whereas CD3-ε appeared exclusively in the included fractions (Fig. 2). In agreement with the results in Fig. 1, approximately 10% of CD4 appeared in the void volume fractions. This material disappeared from the void in the presence of octyl-glucoside or by incubating the extracts at 37°C (not shown), two standard procedures used to solubilize glycolipid-enriched membranes (15). This indicates that the insoluble CD4 correponds to CD4 present in glycolipid-enriched membranes and not to denatured CD4 aggregates.

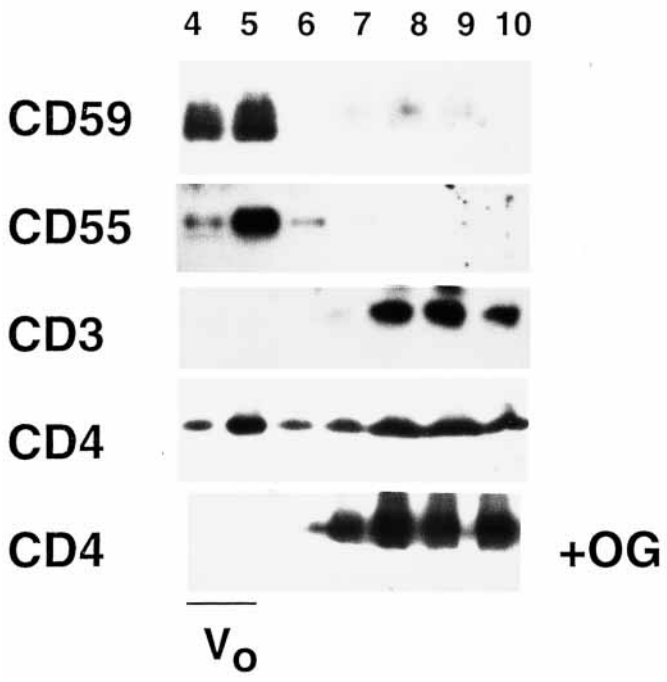

Fig. 2. **Isolation of detergent-insoluble membranes from HPB-ALL cells by molecular filtration chromatography.** 2×10^7 HPB-ALL cells were extracted at 4°C with 1% Triton X-100 or with 1% Triton X-100 plus 60 mM octyl-glucoside (+OG), centrifuged to remove nuclei, cytoskeleton and protein aggregates, and the postnuclear supernatant was fractionated on a Sepharose 4B column at 4°C. Aliquots from each fraction were subjected to immunoblot analysis with either anti-CD59, anti-CD55, anti-CD4, or anti-CD3ε mAb. Fractions 4 and 5 correspond to the void volume (V_o) of the column.

Segregation of CD4 into different insoluble membrane microdomains

To gain insight into the complexity of the detergent-insoluble membrane fraction, we analyzed the presence of GPI-anchored proteins and CD4 after preclearing with anti-CD3ε, anti-CD59 or anti-CD4 antibodies (Fig. 3A). As CD3ε is excluded from the Triton X-100 resistant membranes (Fig. 2), the amount of CD59, CD55 or CD4 remaining in insoluble membranes after preclearing with anti-CD3ε negative control antibodies was taken as 100% of the corresponding insoluble antigen. Densitometric measurements indicated that approximately 40% of the CD4 present in insoluble membranes was resistant to CD59 preclearing. Conversely, approximately 60% of both GPI-attached CD59 and CD55 proteins remained after CD4 preclearing.

To exclude the possibility that the CD4 present in the CD59-precleared samples could be CD4 solubilized during the assay, we performed a second gel filtration chromatography using either CD3ε- or CD59-precleared insoluble membranes. As shown in Fig. 3B, in both cases CD4 was detected in the void volume fractions indicating that CD4 mostly remained in an insoluble form. The insoluble CD4 remaining after CD59 preclearing was solubilized by 60 mM octyl-glucoside or by heating at 37°C for 30 min (not shown), suggesting that the CD4 in the void volume fractions is present in glycolipid-enriched membranes. Moreover, the amount of CD4 after CD59 preclearing was lower than that in samples precleared with the anti-CD3ε negative control antibodies, corroborating that a fraction of the "insoluble CD4" segregates from the membrane microdomains containing GPI-anchored proteins. As a control for the efficiency of the CD59-preclearing, blots with anti-CD59 antibodies are also shown.

The insoluble membranes with only CD4 are depleted of glycolipid-associated kinase activity

The fact that membrane microdomains containing GPI-anchored proteins are enriched in glycolipid-associated kinase activity led us to investigate the presence of kinase activity in the insoluble membrane fraction containing only CD4. To this end, we used membranes isolated by gel filtration chromatography to carry out preclearing experiments with anti-CD59 or anti-CD4 mAb. Anti-CD3ε mAb was used as a negative control since CD3-ε is not present in Triton X-100-insoluble membranes (Fig. 2). Each of the precleared lysates were then subjected to immunoprecipitation with either anti-CD3ε, anti-CD59 or anti-CD4 mAb, and the immunoprecipitates used for *in vitro* kinase assays to detect the presence of associated kinases. To study only the glycolipid-associated kinases, the assays were done in the presence of iodoacetamide which disrupts the direct association of CD4 with Lck mediated by cysteine residues (11). As shown in Fig. 4, the kinase activity in the samples precleared with anti-CD3ε negative control antibodies and immunoprecipitated with anti-CD4 mAb was lower than that immunoprecipitated with anti-CD59 mAb. This supports the results of Fig. 3 showing that not all the insoluble CD4 is in the same microdomains as the CD59 antigen. Interestingly, CD59 preclearing removed the kinase activity in the CD4 immunoprecipitates, indicating that

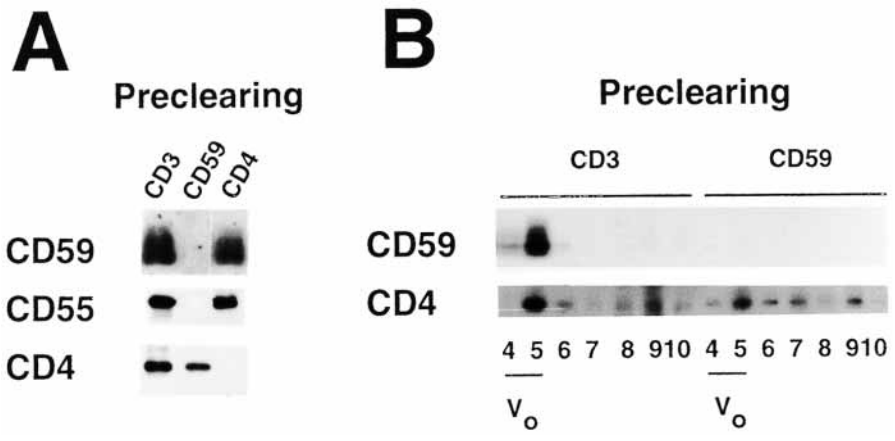

Fig. 3. **Detergent-resistant membrane microdomains containing CD4 segregate from those containing GPI-anchored proteins.** A postnuclear supernatant was obtained using HPB-ALL cells extracted with 1% Triton X-100 at 4°C. After chromatography on a Sepharose 4B column, the insoluble material in the void volume fractions was precleared with either anti-CD59 or CD4 mAb, or with anti-CD3ε mAb as a negative control. **(A) Western blotting analysis of the precleared complexes.** CD3ε-, CD59- or CD4-precleared complexes were subjected to immunoblot analysis with anti-CD59, anti-CD55 or anti-CD4 mAb. **(B) Gel filtration chromatography of extracts precleared with anti-CD59 mAb.** The samples containing the CD59- or CD3ε-precleared material shown in (A) were subjected to a second molecular filtration chromatography. The resulting fractions were analyzed by immunoblotting with either anti-CD59 or anti-CD4 mAb to check that most of the remaining CD4 was not solubilized during the procedure.

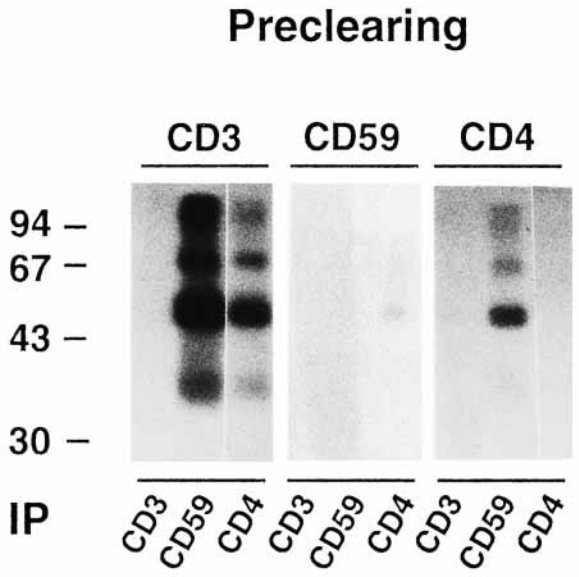

Fig. 4. **CD4-containing insoluble membrane microdomains lacking GPI-anchored proteins are depleted of glycolipid-associated kinase activity.** Precleared lysates were immunoprecipitated with immobilized anti-CD3ε, anti-CD4 or anti-CD59 mAb, and the immunoprecipitates used for *in vitro* kinase assays.

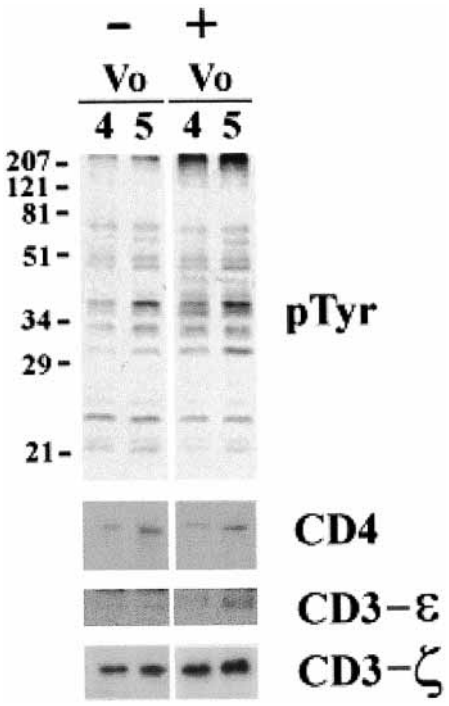

Fig. 5. **Components of the TCR/CD3 complex are recruited into detergent-insoluble membranes upon T-cell activation.** Jurkat cells were incubated with 5 μg/ml of either irrelevant control 9E10 antibodies (−) or anti-CD3 MEM-57 and MEM-92 mAb (+). After 3 min, cells were extracted with 1% Triton X-100 at 4°C and the lysates subjected to chromatography on a Sepharose 4B column. The material in fractions 4 and 5 corresponding to the void volume (Vo) was collected and analyzed by immunoblotting with anti-phosphotyrosine, anti-CD4, anti-CD3-ε or anti-CD3-ζ antibodies. The positions of molecular mass standards are indicated at the left.

the fraction of CD4 in insoluble membranes without CD59 lacks glycolipid-associated kinase activity. Finally, kinase activity was detected in the CD4-precleared samples immunoprecipitated with anti-CD59 antibodies, corroborating that there are membrane microdomains with CD59 and high kinase activity and lacking CD4. These results are in agreement with the preclearing experiments shown in Fig. 3 showing a partial segregation of CD59 and CD4 into different membrane microdomains. Taken together, these results suggest the existence of three distinct types of insoluble membrane microdomains: the first one with both CD59 and CD4; the second type with only CD59; and the third type with only CD4. As determined by densitometric scanning, the first two types account for approximately 85% and 15%, respectively, of the total glycolipid-associated kinase activity present in the CD59-containing insoluble membranes. The insoluble membrane microdomains containing only CD4 display negligible glycolipid-associated kinase activity.

TCR/CD3 components are recruited into the insoluble membrane fraction containing CD4 upon T-cell activation

To begin to address what of the pools of CD4 might participate in activation events triggered by TCR crosslinking, Jurkat cells were incubated with irrelevant anti c-Myc mAb 9E10 or with anti-CD3-ε mAbs. After 3 min of treatment, cells were extracted with 1% Triton X-100 at 4°C and the lysates were subjected in parallel to fractionation on a Sepharose 4B column. The material in the void was collected and analyzed by immunoblot with either anti-phosphotyrosine, anti-CD4, anti-CD3-ε or CD3-ζ antibodies. Fig. 5 shows that TCR crosslinking induced a 4-fold increase in tyrosine phosphorylation of proteins present in insoluble membranes. Moreover, whereas the levels of CD4 were unaffected after activation, small levels of CD3-ε and CD3-ζ were detectable in the insoluble membrane fraction prepared from activated cells but were undetectable in the equivalent fraction from control cells. Thus, the CD4 molecules present in insoluble membranes might joint to newly-recruited TCR/CD3 components to participate in T-cell activation.

Discussion

The existence of membrane microdomains with a lipid composition different from that of the rest of the cellular membranes has been ob-

served in a variety of cell types (5, 23). The high content in both glycolipids and cholesterol in these microdomains provides the basis for their intrinsic resistance to nonionic detergent solubilization (3). In T cells, membranes insoluble in Nonidet P-40 are highly enriched in GPI-anchored proteins and Src-like tyrosine kinases (2). In addition, CD4 and CD8 were also detected in this membrane fraction, whereas the rest of the transmembrane proteins assayed (CD2, CD3, CD5, CD7, etc.) were excluded from these microdomains (11). A different type of membrane microdomain, including many of the antigens solubilized by Nonidet P-40, has been recently isolated using milder detergents such as Brij-58 (24), suggesting a differential sensitivity of the distinct membrane subdomains to detergent solubilization. Even with a given detergent, distinct populations of insoluble membranes have been observed within a single cell type (16–19).

The present study was aimed to address the relationship between CD4 and GPI-anchored proteins in glycolipid-enriched membrane microdomains resistant to Triton X-100 solubilization. Preclearing of insoluble membranes from HPB-ALL cells with different antibodies followed by immunoblotting analysis suggested the existence of three types of insoluble membrane microenvironments: 1) with CD4 but without GPI-attached glycoproteins; 2) with GPI-anchored proteins but without CD4; and 3) with GPI-anchored proteins and CD4. The percentage of CD4 in insoluble membranes lacking GPI-anchored proteins was estimated to be around 40% of the CD4 present in insoluble membranes, and it may account for approximately 4% of total CD4. This percentage seems significant enough to attribute a possible biological relevance to these novel microdomains. Membrane microdomains with CD4, as the standard microdomains with GPI-anchored proteins, are of large size and low density as shown by gel filtration and ultracentrifugation experiments. However, compared with the microdomains containing GPI anchored proteins, the microdomains with only CD4 lack detectable glycolipid-dependent *in vitro* kinase activity. The specific confinement of CD4 within different membrane microenvironments might prevent any lateral interaction of CD4 with other molecules which can not have access to the CD4-enriched microdomains. In addition, the segregation of CD4 into different microdomains might reflect a physical separation of some of the multiple functions performed by CD4 (14). Our results showing the recruitment of TCR/CD3 components in insoluble membranes after T-cell activation is consistent with the hypothesis that the CD4 molecules present in these membranes might participate in the activation events. These results are in agreement with two recent reports showing that membrane compartimentation is required for efficient T-cell activation (24, 25).

In summary, a fraction of the CD4 co-receptor can be found in T cells in detergent-resistant membrane microdomains together with or separated from GPI-attached proteins. These two types of microdomains may possibly contribute to novel aspects of CD4. Placed in a broad context, our results are in agreement with the existence at the T-cell surface of different types of specialized microenvironments with a defined protein and lipid composition (26). The identification and characterization of these microdomains are major goals for the full understanding of the structure and function of the T-cell membrane (24, 27).

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