p56^{lck}, LFA-1 and PI3K but not SHP-2 interact with G_{M1}- or G_{M3}-enriched microdomains in a CD4–p56^{lck} association-dependent manner

Christiane BARBAT*^{†1}, Maylis TRUCY*^{†1}, Maurizio SORICE[‡], Tina GAROFALO[‡], Valeria MANGANELLI[‡], Alain FISCHER*^{†§} and Fabienne MAZEROLLES*^{†2}

*Inserm, U768, Paris, F-75015, France, [†]Université Paris Descartes, Faculté de Médecine René Descartes, Hôpital Necker-Enfants-Malades, 149 rue de Sèvres, Paris, F-75015, France, [‡]Dipartimento di Medicina Sperimentale, Università 'La Sapienza', Viale Regina Elena 324, 00161 Rome, Italy, and [§]Assistance Publique Hôpitaux de Paris (AP-HP), Hôpital Necker-Enfants-Malades, Paris, F-75015, France

We previously showed that the association of CD4 and G_{M3} ganglioside induced by CD4 ligand binding was required for the down-regulation of adhesion and that aggregation of ganglioside-enriched domains was accompanied by transient co-localization of LFA-1 (lymphocyte function-associated antigen-1), PI3K (phosphoinositide 3-kinase) and CD4. We also showed that these proteins co-localized with the G_{M1} ganglioside that partially co-localized with G_{M3} in these domains. In the present study, we show that CD4–p56^{lck} association in CD4 signalling is required for the redistribution of p56^{lck}, PI3K and LFA-1 in ganglioside-enriched domains, since ganglioside aggregation and recruitment of these proteins were not observed in a T-cell line (A201) expressing the mutant form of CD4 that does not bind p56^{lck}. In addition, we show that although these proteins associated in different ways with G_{M1} and G_{M3}, all of the associations were dependent on CD4–p56^{lck} association. Gangliosides could associate with these proteins that differ in affinity binding and could be modified following CD4

signalling. Our results suggest that through these associations, gangliosides transiently sequester these proteins and consequently inhibit LFA-1-dependent adhesion. Furthermore, while structural diversity of gangliosides may allow association with distinct proteins, we show that the tyrosine phosphatase SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2), also required for the down-regulation of LFA-1-dependent adhesion, transiently and partially co-localized with PI3K and p56^{lck} in detergent-insoluble membranes without association with G_{M1} or G_{M3}. We propose that CD4 ligation and binding with p56^{lck} and their interaction with G_{M3} and/or G_{M1} gangliosides induce recruitment of distinct proteins important for CD4 signalling to form a multimolecular signalling complex.

Key words: adhesion molecule, CD4 T-cell, ganglioside, lymphocyte function-associated antigen-1 (LFA-1), phosphoinositide 3-kinase (PI3K), raft.

INTRODUCTION

CD4 signalling, in the absence of TCR (T-cell receptor) engagement, is a critical event in the down-regulation of LFA-1 (lymphocyte function-associated antigen-1)-dependent adhesion between T- and B-cells. We have previously reported that CD4-induced down-regulation requires intermediary proteins between CD4 and LFA-1 such as CD4-associated tyrosine kinase p56^{lck}, PI3K (phosphoinositide 3-kinase) and SHP-2 (Src homology 2 domain-containing protein tyrosine phosphatase 2) [1]. In addition, given the rapid and transient complex of signalling proteins induced by CD4 signalling and signal inhibition by cholesterol removal, we suggested that these proteins were recruited into different membrane compartments [2]. Lateral heterogeneity in the classic fluid-mosaic model of cell membranes indicates that the plasma membrane contains distinct microdomains that are enriched in cholesterol, GPI (glycosylphosphatidylinositol)-linked glycoproteins [3], and glycosphingolipids such as G_{M1} and G_{M3} which are involved in modulating signal transduction [4]. Asymmetric distribution of T-cell rafts containing G_{M1} and G_{M3} glycosphingolipids indicates that rafts have distinct functions and play a role in several signalling processes involving receptors and integrins. Isolation of DRMs (detergent-resistant membranes) has proved to

be a valuable tool for the analysis of lipid rafts and a useful starting point for defining membrane subdomains and their composition [5,6], even though DRMs do not necessarily represent all the rafts in living cells. However, although detergent extraction disrupts lipid–lipid interactions, and lipid–protein interaction, a minor fraction of cell membrane is preserved. Only those proteins that are strongly interacting with highly ordered domains retain their association with lipids and are recovered in DRMs. These DRMs or rafts allow lateral segregation of proteins and provide a mechanism for the compartmentalization of signalling components by concentrating or excluding certain components such as CD45 that inhibit T-cell signalling [7,8]. Thus lipid rafts may function as platforms to control the localization and function of proteins for the formation of multicomponent transduction complexes. The role of lipid rafts in T-cell signalling has also been emphasized given that disruption and/or displacement of signalling molecules abolishes TCR-mediated signalling events [9]. In lymphocytes, G_{M3} and cholesterol, along with the Src-family kinase p56^{lck} and a fraction of the CD4 pool, are acylated by saturated fatty acids and are selectively recovered in the rafts where they associate [10,11]. Mutant versions of p56^{lck} and CD4 that are not palmitoylated and consequently not localized within rafts, do not function in T-cell activation [12,13]. Polarization and lipid raft recruitment

Abbreviations used: Ab, antibody; AU, arbitrary units; CTxB, cholera toxin; DRM, detergent-resistant membrane; GAMlg, goat anti-mouse Ig; HLA, human leucocyte antigen; HPTLC, high-performance TLC; HRP, horseradish peroxidase; LFA-1, lymphocyte function-associated antigen-1; mAb, monoclonal Ab; PI3K, phosphoinositide 3-kinase; PDK1, phosphoinositide-dependent kinase-1; PNS, post-nuclear supernatant; RN, relative number; SHP-2, Src homology 2 domain-containing protein tyrosine phosphatase 2; TCR, T-cell receptor; TRITC, tetramethylrhodamine β -isothiocyanate.

¹ These authors contributed equally to this work.

² To whom correspondence should be addressed (email mazerol@necker.fr).

to the receptor engagement site in immune cells following solid-phase engagement of the CD4 molecule alone, and subsequent redistribution of different proteins such as integrins, requires p56^{lck} signalling [14]. Raft localization of integrins appears to be correlated with integrin activity, since inactive integrins such as LFA-1 are tethered away from lipid rafts by cytoskeletal restraints [15]. Other proteins such as SHP-2 and PI3K can affect both within raft-integrin localization and function. SHP-2 associates with adhesion molecules [16] and SHP-2 partitioning in raft domains triggers integrin-mediated signalling [17]. SHP-2 also associates with F-actin [18] and plays a role in cytoskeletal organization, cell adhesion and migration [19]. PI3K can also be recruited in raft domains following activation, and disruption of these domains inhibits the PI3K pathway [20]. PI3K, through its different associations with protein kinase C ζ , RhoA or cytohesin-1, is also important for integrin regulation [21,22] as well as for the polar redistribution of LFA-1 induced by chemokines [23].

We have previously reported that lipid raft integrity is required for the down-regulation of Ag-independent LFA-1-dependent adhesion induced by CD4 ligands, and more precisely by an anti-CD4 Ab (antibody; 13B8.2) that specifically binds the N-terminal CD4 domain (D1) [24]. The fact that CD4 triggering induces aggregation of G_{M1}- and G_{M3}-enriched raft domains that are partially co-localized suggests that G_{M1} and G_{M3} may also be localized in distinct domains and may associate with distinct proteins. Raft involvement in CD4-triggered events has been strengthened by the results showing that the CD4–G_{M3} association induced by CD4 ligand binding was required for the down-regulation of LFA-1-mediated adhesion [24]. Raft aggregation was accompanied by transient co-localization of LFA-1, PI3K and CD4 in these domains. We investigate here whether LFA-1, PI3K and SHP-2 co-localize and associate with gangliosides after CD4 ligation and whether gangliosides act as intermediary partners for the sequential association of these different proteins. In addition, since p56^{lck} associated with CD4 was also required for the down-regulation of Ag-independent LFA-1-dependent adhesion induced by CD4 ligands [25], we also investigate whether CD4–p56^{lck} association is also required for the LFA-1, PI3K and SHP-2 co-localization and association with gangliosides.

MATERIALS AND METHODS

Abs and reagents

The following Abs were used: 13B8.2, 25.3 [IgG1, anti-CD4 and anti-LFA-1 α mAbs (monoclonal Abs) respectively], anti-HLA (human leucocyte antigen) class I Ab from Immunotech, anti-p85-PI3K polyclonal Ab (Upstate Biotechnology), anti-p56^{lck} polyclonal Ab and anti-SHP-2 mAb (Santa Cruz Biotechnology). F(ab)₂ GAMIg (goat anti-mouse Ig; Jackson ImmunoResearch Laboratories) was used for cross-linking experiments. For confocal immunofluorescence experiments, the following Abs were used: TRITC (tetramethylrhodamine β -isothiocyanate)-conjugated GAMIg and FITC-conjugated sheep anti-rabbit IgG (Jackson ImmunoResearch Laboratories), and anti-G_{M1} and anti-G_{M3} mAbs (Seikagaku).

Cell cultures

The A201 T-cell line was a CEM-derived T-cell line, transfected with wild-type CD4 cDNAs (A201-CD4), or with a mutated form of CD4 cDNAs (A201-2C > A) in which the two cysteine residues required for association with p56^{lck} were replaced by two alanine

residues [25]. These T-cell lines normally express CD4 and equivalent amounts of active p56^{lck} at the cell surface [25]. No association between the mutated form of CD4 and p56^{lck} was found in A201-2C > A. Cells were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10 % (v/v) fetal calf serum, 2 mM L-glutamine and 0.5 mg/ml of G418 (Life Technologies).

Isolation of DRMs by flotation experiments

Optiprep gradient analysis was performed according to a previously described method with some modifications [26]. Briefly, as previously reported [24], A201-CD4 T-cell lines were starved overnight in serum-free medium; 25×10^6 cells were then incubated with anti-CD4 Ab for 20 min at 4 °C, washed and incubated for the indicated time at 37 °C with a GAMIg. Cells were then lysed on ice for 15 min in 900 μ l of lysate buffer A (50 mM Tris/HCl, pH 7.4, 110 mM NaCl, 10 mM EGTA, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 2 μ g/ml aprotinin and 1 mM PMSF) without detergent and sonicated gently (5 s bursts, 5 W; Branson sonifier 250). After centrifugation at 800 g for 5 min at 4 °C, the PNS (post-nuclear supernatant) was incubated with 1 % Brij 58 at 4 °C for 1 h. DRMs were isolated by ultracentrifugation at 28 000 rev./min for 4 h at 4 °C in a SW41 rotor (Beckman Instruments), in a 40 %/30 %/5 % Optiprep density gradient (Sigma). Seven fractions were collected from the top of the tube. F1 corresponds to the top of the gradient. The low-density fractions 2, 3 and 4 contained detergent-insoluble raft fractions enriched in gangliosides. As expected, transferrin receptor that does not reside in rafts was only detected in fractions 6 and 7 that contained detergent-soluble membrane. Normalized protein amounts for each fraction were determined using the Bio-Rad kit with BSA as the standard. Each fraction was then immunoprecipitated with specific Abs and analysed by SDS/8 % PAGE and Western blotting as previously described [24]. Proteins were visualized using an ECL[®] (enhanced chemiluminescence) detection system (Amersham Biosciences) with an anti-rabbit or anti-mouse Ig coupled with HRP (horseradish peroxidase) as the secondary Ab (Amersham Biosciences). G_{M1} and G_{M3} gangliosides were detected using HPTLC (high-performance TLC) [1], as reported below. The ganglioside extract was split into two aliquots. The first one was run on silica gel 60 HPTLC plates (Merck) and stained with resorcinol to detect G_{M3}. The second one was run on HPTLC aluminium-backed silica gel 60 (20 $\times$ 20) plates (Merck). The plates were immunostained with 0.5 μ g/ml of CTxB (cholera toxin)–HRP for 1 h to detect G_{M1} at room temperature (25 °C). Immunoreactivity was assessed by chemiluminescence.

Ganglioside detection by HPTLC

A201-CD4 or A201-2C > A T-cell lines were incubated overnight at 37 °C in serum-free medium before incubation with anti-CD4 Abs. Cells (40×10^6) were incubated for 20 min at 4 °C with anti-CD4 Ab and then washed and incubated for the indicated time at 37 °C with a GAMIg. Cells were then lysed on ice for 20 min in 900 μ l of lysate buffer A supplemented with 1 % Nonidet P40, and clarified by centrifugation at 12 000 g for 15 min. The same amount of each PNS (4 mg) was immunoprecipitated with specific Abs and analysed for the presence of the gangliosides by HPTLC. Gangliosides were extracted twice in chloroform/methanol/water (4:8:3, by vol.) and subjected to Folch partition by the addition of water, resulting in a final chloroform/methanol/water ratio of 1:2:1.4. The upper phase, containing polar glycosphingolipids, was purified of salts and low-molecular-mass contaminants

Figure 1 Role of CD4-p56^{lck} association in G_{M1} and G_{M3} distribution following CD4 ligation

A201-CD4 (**A**, **C**) and A201-2C>A (**B**, **C**) T-cell lines were incubated at 4°C for 15 min with either anti-CD4 Ab (A) or an anti-transferrin receptor Ab used as a control (Ct) and then either cross-linked with GAMlg during the time indicated or incubated with GAMlg alone (NA or G-) at 37°C for 10 min. G_{M1} and G_{M3} were detected with appropriate Abs and co-localizations are shown in the merged panels. A 3-fold enlargement (3X) of single cells is shown in the merged panels (marked with an arrow). Results shown are from one representative experiment out of ten. In (**C**), following CD4 cross-linking, cells were washed, lysed and the presence of the gangliosides was detected by HPTLC. The standard gangliosides are indicated (St).

using Bond Elut C₁₈ columns (Superchrom). The eluted glycosphingolipids were dried and separated by HPTLC using silica gel 60 HPTLC plates (Merck). Chromatography was performed in chloroform/methanol/aq. 0.25 % KCl (5:4:1, by vol.). The plates were stained with resorcinol (ganglioside-specific stain) or with cholera toxin for G_{M1}, and anti-G_{M3} Ab [1]. Quantification was carried out by densitometric scanning analysis using a Mac OS 9.1 (Apple Computer International) and NIH (National Institutes of Health) Image 1.61 software. The amount of immunoprecipitated protein was checked by control Western blotting with the Abs used for specific immunoprecipitation.

Co-localization experiments using scanning confocal microscopy

As described previously [24], after activation with cross-linked anti-CD4 Ab and saturation of the free sites with GAMlg incubation at 4°C, cell suspensions containing 8×10^4 cells/slip were layered on to poly(L-lysine)-coated coverslips for 45 min at room temperature. Immunofluorescence staining of cell surface molecules was performed with the appropriate mAbs in the absence of

permeabilizing agent, followed by FITC- or TRITC-anti-mouse IgG1 or anti-rabbit secondary Abs. Intracellular proteins were stained for 1 min in 0.05 % saponin-permeabilized cells using the appropriate mAbs. We have previously checked that labelled GAMlg does not reveal anti-CD4 Ab [24]. Confocal microscopy was performed on a Zeiss LSM-510 confocal microscope. Images were acquired using the maximum signal detection setting below the saturation limit of the detector.

RESULTS

The CD4-p56^{lck} association is required for the aggregation of G_{M1}(+) and G_{M3}(+) DRMs induced by anti-CD4 Ab

We have previously shown that CD4 ligand binding specifically induces CD4 aggregation and partial co-localization of G_{M1}- and G_{M3}-enriched domains [24]. Integrity of raft domains and the CD4-p56^{lck} association were required for the down-regulation of LFA-1-dependent adhesion induced by CD4 ligand binding [24]. We have therefore investigated whether the CD4 ligand induces

Figure 2 Role of CD4–p56^{lck} association in the co-localization and association of LFA-1 with G_{M1} and G_{M3} gangliosides following CD4 ligation

A201-CD4 and A201-2C > A T-cell lines (5×10^4 in **A**, 40×10^6 in **B**) were incubated at 4 °C for 15 min with either an anti-CD4 Ab (A-) or an anti-transferrin receptor Ab (A-; Ct-2) or an anti-HLA-I Ab (B; Ct), used as a control, and then cross-linked with GAMlg during the indicated time or treated with GAMlg alone at 37 °C for 20 min (NA in **A**, G in **B**). In (**A**), LFA-1, G_{M1} and G_{M3} are shown with the appropriate Abs; co-localizations are shown in merged panels. The right column shows a 3-fold enlargement (3X) of single cells marked with an arrow in merged panels. Results shown are from one representative experiment out of eight. In (**B**), proteins were immunoprecipitated with an anti-LFA-1 Ab and analysed for the presence of the gangliosides by HPTLC. Standard gangliosides are indicated (St). The amount of LFA-1 immunoprecipitated was verified with an anti-LFA-1 immunoblot.

ganglioside-enriched domain aggregation in a CD4–p56^{lck}-dependent manner. We observed that G_{M1}- and G_{M3}-enriched domain aggregation, detected following CD4 ligation (Figure 1A), was dependent on CD4–p56^{lck} association, since it was not observed in A201-2C > A, an A201-T-cell line transfected with a mutated form of CD4 that does not bind p56^{lck} (Figure 1B). Patching was not observed with a control Ab specific to transferrin receptor (Figures 1A and 1B, Ct). HPTLC showed that both T-cell lines expressed the same amount of ganglioside under the tested conditions (Figure 1C).

CD4 ligation induces the redistribution of LFA-1 and PI3K with G_{M1} and G_{M3} in a CD4–p56^{lck} association-dependent manner

We have previously reported that PI3K and LFA-1 partially co-localized with G_{M1} and G_{M3} in DRMs following CD4 triggering [24]. In the present study, we investigated whether CD4–p56^{lck} association was also required for these co-localizations. Distribution of LFA-1 and PI3K, which is uneven and punctuated over the plasma membrane following CD4 cross-linking, appeared distributed all over the membrane without detectable clustering in the A201-2C > A T-cell line; this was also observed in both T-cell lines in the absence of CD4 triggering (NA) and in the control Ab-treated cells (Ct; Figures 2A and 3A). These immunofluorescence

experiments also showed that the co-localizations of G_{M1}, LFA-1 and PI3K respectively and of G_{M3} and PI3K were partial but stable over a 20 min period. Co-localization of LFA-1 with G_{M3} was more dominant after a short period of incubation with anti-CD4 Ab (2 min) than following longer incubation (Figure 2A, left panel).

To determine whether LFA-1 and PI3K interact with G_{M1} and G_{M3} gangliosides following CD4 cross-linking, cell-free lysates from anti-CD4-treated cells were immunoprecipitated with an anti-LFA-1 mAb (Figure 2B, and Supplementary Figure S1A at <http://www.BiochemJ.org/bj/402/bj4020471add.htm>) or an anti-PI3K Ab (Figure 3B and Supplementary Figure S1B), and gangliosides were detected by HPTLC as previously reported [1]. Immunoprecipitation with an anti-LFA-1 mAb (Figure 2B and Supplementary Figure S1A) and gangliosides detected by HPTLC showed a faint G_{M3} band but no G_{M1} band following control incubation with GAMlg (Figure 2B). In contrast, after 2 min of CD4 cross-linking, two main resorcinol-positive bands co-migrating with G_{M3} [82 ± 10 arbitrary units (AU); Supplementary Figure S1A] and, to a lesser extent, with G_{M1} (57 ± 5 AU; Supplementary Figure S1A) were detected. Co-precipitation of G_{M3} with LFA-1 rapidly decreased (30 ± 5 and 24 ± 2 AU after 10 and 20 min respectively; Supplementary Figure S1A). In contrast, co-precipitation of G_{M1} with LFA-1 was lower but stable and

Figure 3 Role of CD4-p56^{lck} association in the co-localization and association of PI3K with GM₁ and GM₃ gangliosides following CD4 ligation

A201-CD4 and A201-2C > A T-cell lines (5×10^4 in **A**, 40×10^6 in **B**) were incubated at 4 °C for 15 min either with an anti-CD4 Ab (A-) or with an anti-transferrin receptor Ab (A) and an anti-HLA-1 Ab (B) used as a control (Ct) and then cross-linked with GMIg for the indicated time or treated with GMIg alone at 37 °C for 20 min (NA in **A**, G in **B**). In (**A**), PI3K, GM₁ and GM₃ were detected with appropriate Abs and co-localizations are shown in merged panels. The right column depicts a 3-fold enlargement (3X) of single cells stained in the merge. Results shown are from one representative experiment out of six. In (**B**), proteins were immunoprecipitated with an anti-PI3K Ab and analysed for the presence of the gangliosides by HPTLC. Standard gangliosides are indicated (St). The amount of PI3K immunoprecipitated was verified with an anti-PI3K immunoblot.

decreased more slowly (Supplementary Figure S1A). Confocal microscopy analysis also showed that the LFA-1-GM₁ interaction was more stable than the LFA-1-GM₃ interaction. Co-localization in patches (Figure 2A) and co-precipitation of LFA-1 and gangliosides (Figure 2B) both required CD4-p56^{lck} association since neither was detected in the A201-2C > A T-cell line. The same amount of LFA-1 was immunoprecipitated under the different conditions used in A201-CD4 and A201-2C > A T-cell lines as shown with the anti-LFA-1 immunoblot (Figure 2B).

In the PI3K immunoprecipitates (Figure 3B and Supplementary Figure S1B), resorcinol-positive bands were not detected following control activation (Ct), whereas GM₁ and, to a lesser extent, GM₃ were detectable following activation with the anti-CD4 Ab. Although co-precipitation was stable for 20 min, GM₃ co-precipitation with PI3K was always lower than with GM₁ (Figure 3B and Supplementary Figure S1B). No ganglioside bands were detected in the extracts from the PI3K immunoprecipitates at the surface of the A201-2C > A T-cell line (Figure 3B), indicating that PI3K interaction with lipid rafts also requires CD4-p56^{lck} association. The same amount of PI3K was immunoprecipitated under the different conditions used in A201-CD4 and A201-2C > A T-cell lines as shown with the anti-PI3K immunoblot (Figure 3B).

Anti-CD4 Ab induces redistribution of p56^{lck} and SHP-2 with GM₁ and GM₃ in a CD4-p56^{lck} association-dependent manner

The above results indicated that CD4-associated-p56^{lck} is required for the aggregation of GM₁ and GM₃, and the co-localization of PI3K and LFA-1 after CD4 signalling, and that p56^{lck} may also be redistributed with these gangliosides. Scanning confocal microscopy revealed p56^{lck} clustered in patches co-stained with anti-GM₁ and anti-GM₃ Abs (Figure 4A). All of the GM₃-containing domains but only some of the GM₁-containing domains co-localized with p56^{lck} (Figure 4A). Aggregation of p56^{lck} induced by CD4 ligand binding was not detected in the A201-2C > A T-cell line, indicating that aggregation was dependent on CD4-p56^{lck} association (Figure 4A). Patching was not detected in either of the T-cell lines following cross-linking with a control Ab (Figure 4A, Ct-2). However, CD4 cross-linking led to a transient increase in co-precipitation of GM₃ with p56^{lck}, which was also dependent on CD4-p56^{lck} association since it was not detected in the A201-2C > A T-cell line (Figure 4B). TLC immunostaining analysis (a more sensitive method than resorcinol staining) using CTxB that specifically binds GM₁ and anti-GM₃Ab for GM₃ detection (Figure 4C) revealed co-precipitation between p56^{lck} and GM₁. Ganglioside association was not detected following

Figure 4 Co-localization and association of p56^{lck} with G_{M1} and G_{M3} induced by CD4 ligation are dependent on CD4–p56^{lck} association

A201-CD4 and A201-2C > A T-cell lines (5×10^4 in **A**, 40×10^6 in **B**) were incubated at 4°C for 15 min either with an anti-CD4 Ab (A-) or an anti-transferrin receptor Ab (**A**) and an anti-HLA-I Ab (**B**) used as a control (Ct) then cross-linked with GAMlg for the indicated time, or treated with GAMlg alone at 37°C for 20 min (NA in **A**, G in **B**). In (**A**), localization of p56^{lck} with G_{M1} and G_{M3} was detected with appropriate Abs. Co-localizations are shown in the merged panels. Results shown are from one representative experiment out of six. In (**B**), proteins were immunoprecipitated with an anti-p56^{lck} Ab and analysed for the presence of the gangliosides by HPTLC staining analysis by resorcinol (**B**), or by CTxB for G_{M1} detection, and anti-G_{M3} Ab (**C**). Standard gangliosides are indicated (St). Densitometric analysis of G_{M1} and G_{M3} co-precipitated with p56^{lck} was indicated in arbitrary units (AU). The amount of p56^{lck} immunoprecipitated was verified with an anti-p56^{lck} immunoblot.

incubation with a control Ab (Figure 4B, Ct). The same amount of p56^{lck} was immunoprecipitated in the HPTLC (Figure 4B) and TLC (Figure 4C) analyses in both T-cell lines.

SHP-2 tyrosine phosphatase was previously reported to be required for the formation of a multi-protein complex with PI3K induced by CD4 ligand binding [2]. In the present study, CD4 triggering induced very rapid redistribution of SHP-2 with both G_{M1} and G_{M3} (Figure 5A). After CD4 cross-linking for 20 min, SHP-2 was distributed predominantly in the cytoplasm, and was less aggregated in patches on the plasma membrane, indicating that SHP-2 only transiently localized in these ganglioside-enriched domains. In contrast, G_{M1} and G_{M3} aggregation was maintained, indicating that the delocalization of SHP-2 from these domains was not due to disruption of the ganglioside-enriched domains. The rapid and transient localization of SHP-2 in G_{M1}- and G_{M3}-enriched domains was also dependent on CD4–p56^{lck} association, since SHP-2 aggregation was not detected in the A201-2C > A T-cell line (Figure 5A). Patching was not detected following incubation with a control Ab (Figure 5A, Ct-2). However, co-precipitation between SHP-2 and G_{M1} and G_{M3} was not detected, suggesting that either SHP-2 does not interact directly with these gangliosides or, alternatively, the affinity of these interactions was too low to be detected under immunoprecipitation conditions (Figure 5B).

Anti-CD4 Ab induces distinct interactions between p56^{lck}, SHP-2 and PI3K in DRMs

We then investigated whether p56^{lck} co-localized and interacted with SHP-2 and PI3K in DRMs. Immunofluorescence analysis

showed that p56^{lck} did not co-localize with SHP-2 and PI3K in the same manner. Indeed, CD4 cross-linking induced a transient co-localization of p56^{lck} with SHP-2 (Figure 6A), whereas the co-localization of p56^{lck} with PI3K appeared stable for up to 20 min (Figure 7A). These observations were consistent with those described above, showing the transient localization of SHP-2 in raft domains (Figure 5A), in contrast with PI3K (Figure 3A) and p56^{lck} (Figure 4A). Partial co-localization between SHP-2 and PI3K was observed (Figure 6B). Patching was not observed following incubation with a control Ab (Figures 6A, 6B and 7A).

The G_{M1}⁺ and G_{M3}⁺-enriched DRMs containing the isolates from activated and unstimulated T-cells obtained by Optiprep density gradient centrifugation and ultracentrifugation were mainly detected in fractions F2–F4 (Figure 6C, top panel). F1 corresponds to the top of the gradient, and the detergent-soluble membrane was detected in fractions F6 and F7. Neither G_{M1} nor G_{M3} were detected in F1, F6 and F7 (Figure 6C). p56^{lck} (Figure 6C, bottom panel, and Supplementary Figure S2A at <http://www.BiochemJ.org/bj/402/bj4020471add.htm>) was detected in the immunoprecipitates from DRM fractions F2–F4 and one of the detergent-soluble fractions (F6) in the absence of, or following, CD4 cross-linking. In contrast, SHP-2 was mainly detected in detergent-soluble fraction (F6) and DRM fraction 4 in the absence of CD4 cross-linking (Figure 6D and Supplementary Figure S2B). Following CD4 cross-linking for 2 min, SHP-2 was predominantly detected in F3 [RN (relative number)=4, Figure 6C and Supplementary Figure S2B] and F4 DRM-containing fractions (RN=2.5, Figure 6C; RN=5, Supplementary Figure S2B). Following longer CD4 ligation, SHP-2 was detected in all of the tested fractions (Supplementary Figure S2B). However, SHP-2 did not co-precipitate with p56^{lck} in any of the DRM fractions with

the exception of the detergent-soluble fraction F6 (Figure 6C, and Supplementary Figure S3A at <http://www.BiochemJ.org/bj/402/bj4020471add.htm>). Similarly, SHP-2 only co-precipitated with PI3K (Figure 6D and Supplementary Figure S3B) in detergent-soluble membrane fraction F6, as previously reported under other conditions [2]. Co-precipitation between SHP-2 and PI3K was not detected in any of the DRM fractions (Figure 6D and Supplementary Figure S3B). In contrast, PI3K was detected in all of the fractions following CD4 ligation (Supplementary Figures S2C and S3B) as expected, and was also co-precipitated with p56^{lck} in all of the fractions (Figure 7B and Supplementary Figure S3C).

Overall, these results indicate that CD4 ligand binding induces colocalization and aggregation of several proteins involved in CD4

© 2007 Biochemical Society

Figure 6 Distribution and association of SHP-2 with p56^{lck} and PI3K in G_{M1}- and G_{M3}-containing microdomains following anti-CD4 Ab incubation

A201-CD4 T-cell lines (5×10^4 in **A** and **B**, 25×10^6 in **C** and **D**) were incubated for 15 min at 4 °C either with an anti-CD4 Ab or an anti-transferrin receptor Ab used as a control (Ct), and then cross-linked with F(ab)₂ GAMlg during the indicated time at 37 °C, or treated with GAMlg alone (NA and G-20 min). In (**A**, **B**), p56^{lck}, SHP-2 and PI3K are detected with appropriate Abs and co-localizations are shown in merged panels. The 3-fold enlargement (3X) of single cells is marked with an arrow in merged panels. In (**C**, **D**), raft-containing fractions were collected from cells that were activated, lysed and subjected to Optiprep density gradient (F1: top of the gradient; F2–F4: raft fractions; F6 and F7: fractions containing detergent-soluble membrane). G_{M1} was detected by HPTLC immunostaining using a CTxB–HRP conjugate and G_{M3} was detected by HPTLC followed by resorcinol staining. Proteins isolated from the different fractions were immunoprecipitated with an anti-p56^{lck} Ab (**C**) or an anti-SHP-2 Ab (**D**) and then detected by immunoblotting with an anti-p56^{lck} Ab (**C**) or an anti-SHP-2 (**D**) or an anti-PI3K (**D**). Control Ab (ip Rb) was used for the non-specific immunoprecipitation. Results shown are from one representative experiment out of six. Proteins are indicated in each panel. Quantification of the gangliosides detected in each fraction [indicated in arbitrary units (AU)], and the intensity of each band detected by anti-SHP-2, anti-p56^{lck} or anti-PI3K-specific Abs were calculated using NIH Image 6.2 software. Relative number (RN) from each fraction (**C**, **D**) represents the increased factor of the different immunoprecipitated proteins (p56^{lck} or SHP-2) and co-precipitated proteins (SHP-2 or PI3K) in activated cells over unstimulated cells as compared with the respective GAMlg control (G-20 min).

results appear to contrast with those reported by Fragoso et al. [12] showing that association between CD4 and p56^{lck} is not essential for CD4-induced lipid raft aggregation. The most likely explanation for this difference could be the use of an anti-CD4 Ab (OKT4) that binds to the region of CD4 (D4) proximal to the transmembrane domain by Fragoso's team, whereas we used the 13B8.2, which recognizes an epitope located within the first N-terminal domain (D1). The signal induced by these two Abs differs because only anti-CD4 Abs binding to the D1 CD4 domain (such as Leu3a, OKT4a and 13B8.2) inhibit Ag-independent LFA-1-dependent T-cell adhesion on B-cells [28]. In our study, inhibition was not detected with other anti-CD4 Abs, such as OKT4 (F. Mazerolles, unpublished work). In addition, the pattern of tyrosine-phosphorylated proteins following binding of either OKT4- or D1 domain-specific ligands is distinct [29,30]. Fragoso et al. [12] observed enhanced tyrosine phosphorylation of several proteins induced by OKT4 binding in the absence of association between CD4 and p56^{lck} in these mutant lines, showing that this setting differs from ours. Moreover, as OKT4a and Leu3a epitopes are required for G_{M1} signalling in contrast with OKT4 [31], it appears that cross-linking of distinct CD4 epitopes induces different signalling and aggregation of raft domains in a CD4–p56^{lck} association-dependent or -independent manner. Since 13B8.2 binds to the HLA class II interactory domain of CD4, it may be a more appropriate physiological model for inducing CD4 aggre-

gation than OKT4. Furthermore, our results are consistent with reports showing that in the absence of p56^{lck}, lipid rafts do not form caps following CD4 engagement, so CD4 ligation may provide a sufficient signal to induce aggregation of lipid rafts in p56^{lck} (+) Jurkat cells in the absence of TCR engagement [14].

We also show that p56^{lck} totally co-localized with G_{M3}-containing domains in a CD4–p56^{lck} association-dependent manner, while interaction between p56^{lck} and G_{M1} was less intense. Since CD4 associates with G_{M3} [32], CD4 appears to be the main intermediary between p56^{lck} and G_{M3}. On the contrary, as CD4 does not associate with G_{M1} in a similar fashion, additional partners are likely to be inserted between G_{M1} and the CD4–p56^{lck} complex (as proposed in the model depicted in Figure 8). Interestingly, the association between p56^{lck} and G_{M1} and G_{M3} gangliosides did not persist after prolonged CD4 cross-linking (20 min), whereas p56^{lck} remained localized in the DRM domains, indicating that p56^{lck} can persist in rafts without association with gangliosides. The localization of p56^{lck} in lipid rafts in the absence of TCR activation is controversial. In unstimulated cells, most of the p56^{lck} co-localizes with CD4 outside of lipid rafts and translocates into raft domains upon antigen stimulation [12]. Raft integrity has been shown to be important for p56^{lck} activity [33]. On the other hand, others have reported that p56^{lck} localizes mainly in lipid rafts [32,34]. Our results support the view that p56^{lck} can localize within rafts in the absence of antigen presentation, but is further recruited

Figure 7 Distribution and association of p56^{lck} with PI3K in G_{M1}- and G_{M3}-containing microdomains following anti-CD4 Ab incubation

A201-CD4 T-cell lines were similarly treated as described in Figure 6. In (A), p56^{lck} and PI3K are detected with appropriate Abs and co-localizations are shown in merged panels. A 3-fold enlargement (3X) of single cells marked with an arrow is shown in merged panels. In (B), as described in Figure 6, proteins were isolated from the seven fractions collected by Optiprep density gradient and ultracentrifugation and immunoprecipitated with an anti-p56^{lck} Ab and then detected with an anti-p56^{lck} Ab or with an anti-PI3K. Control Ab (ip Rb) was used as a non-specific immunoprecipitation control. Results shown are from one representative experiment out of six. Proteins are indicated in each panel. Relative number (RN) in each fraction represents the ratio of p56^{lck} and PI3K immunoprecipitated in activated cells over unstimulated cells as compared with the respective GAMlg control (G-20 min).

and clustered in these domains following CD4 engagement. They also confirm that CD4 ligation plays a critical role in the recruitment of signalling proteins to rafts and may facilitate cellular reorganization by recruiting adhesion and signalling molecules [14].

We also provide evidence that CD4-p56^{lck} association is required for the recruitment of LFA-1, PI3K and also SHP-2, which is another partner involved in the down-regulation of LFA-1-dependent adhesion induced by CD4 ligand binding [2]. This result supports the hypothesis that CD4-associated p56^{lck} could induce the recruitment of adhesion and signalling molecules in DRMs that are likely to concentrate these proteins in signalling platforms [12]. Our finding that LFA-1 and PI3K were not always co-precipitated with the same gangliosides during CD4-p56^{lck}-induced signalling suggests that these proteins move to different domains containing G_{M1} and/or G_{M3} gangliosides which are not always co-localized. However, spatial resolution would be necessary to differentiate G_{M1}- or G_{M3}-containing domains. It is possible

that prolonged CD4 ligation induced a modification of affinities between proteins and gangliosides and cannot be excluded. Indeed, PI3K did not appear to move from the DRMs during CD4 ligation. It is likely that PI3K indirectly associates with G_{M3} via the p56^{lck}-CD4 complex because CD4 cross-linking also induced association between PI3K and p56^{lck} in the G_{M3}-enriched fractions. The fact that PI3K was less associated with G_{M3} than with G_{M1} suggests that the PI3K isoforms (α and β , indistinguishable from the Ab we used) have a distinct affinity for these gangliosides. On the other hand, LFA-1 partially and stably co-localized with G_{M1}, without notable modification after longer CD4 ligation, while the co-precipitation of LFA-1 with G_{M1} only slowly decreased. In addition, LFA-1 totally co-localized with G_{M3} after a short period of CD4 ligation, followed by a decrease in co-localization. This decrease was correlated with a rapid decrease in LFA-1/G_{M3} co-precipitation, suggesting a possible modification of affinity between these molecules. This modification can also induce a modification of LFA-1 localization in distinct ganglioside-enriched-domains. A similar raft segregation of membrane-receptor redistribution has also been described in migrating cells [35,36]. Integrins co-localized with G_{M1}-enriched rafts redistributed to the uropod, whereas PI3K co-localized with G_{M3}-enriched rafts redistributed to the leading edge. As the G_{M3}-CD4 association is required for CD4 signalling [32,37] leading to the down-regulation of the LFA-1-dependent adhesion induced by CD4 ligation [24], G_{M3} could be a transducer of transmembrane signalling and could play a role in cell adhesion regulation by triggering partners in a multi-protein complex formation [38]. The molecular basis of these ganglioside-protein interactions remains to be determined. Indeed, isoforms of PI3K and LFA-1 are not acylated by saturated fatty acids such as p56^{lck} or CD4 to partition into rafts. Thus we suggest that, in addition to p56^{lck}, other unidentified proteins moving in lipid rafts (X and Y in the model) are also involved in these interactions [15]. LFA-1 may bind cytohesin-1, which binds D3 phospholipids that are enriched in rafts, and other partners participating in the down-regulation of LFA-1 adhesion [2]. This localization in rafts would allow LFA-1 to have a stronger affinity for its ligand and enhance its stability, and increase the activity of PI3K. SHP-2, which associates with PI3K outside of rafts following CD4 binding [2], may be another intermediate regulator of Src kinase activation due to its localization in raft domains [39]. However, although CD4 ligation induces rapid and transient SHP-2 localization in a CD4-p56^{lck} association-dependent manner, direct interaction between SHP-2 and gangliosides or with p56^{lck} and PI3K was not detected. Nevertheless, SHP-2 was co-precipitated with PI3K and p56^{lck} after CD4 ligation in detergent-soluble membrane. The absence of co-precipitation in rafts suggests a possible alternative role for SHP-2 and other partners. We recently observed that outside of raft domains, SHP-2 can associate with the serine kinase PDK1 (phosphoinositide-dependent kinase-1), a specific effector of PI3K that also binds D3 phospholipids (F. Mazerolles and M. Trucy, unpublished work). Thus it is possible that PDK1 also migrates to rafts with SHP-2 and could be the intermediary between PI3K and SHP-2. This hypothesis is under investigation.

In conclusion, these results further support our previous findings of the key role of CD4-p56^{lck} association in CD4 signalling. Indeed, this association, which is important for p56^{lck} and PI3K activities induced by CD4 ligation [25,29], appears to be required for the redistribution of these kinases in ganglioside-enriched domains and co-localized with LFA-1 and SHP-2 that is involved in the regulation of adhesion. Our results also suggest that gangliosides associate with distinct proteins that probably have different affinity binding, and can be modified following CD4 signalling. Through these associations, gangliosides could transiently

Figure 8 A model for the distinct membrane associations between LFA-1, PI3K, p56^{lck} and SHP-2 induced by CD4 ligation, in a CD4-p56^{lck}-dependent manner

In the absence of CD4 ligation (0), LFA-1 is mainly localized in detergent-soluble domains of the membrane (Mb) and PI3K is associated with p56^{lck} in ganglioside-enriched microdomains or DRMs, without association with GM₁, or with GM₃, in contrast with p56^{lck}. After CD4 cross-linking for 2 min (αCD4 + G-2 min), LFA-1 has migrated into raft domains and is mainly associated with GM₃ (directly or via the hypothetical intermediate protein Y) and with GM₁ but to a lesser extent (directly or via the hypothetical intermediate protein X). In contrast, PI3K is mainly associated with GM₁ (directly or via the hypothetical intermediate protein X), and its association with p56^{lck} increases. After CD4 cross-linking for 20 min (αCD4 + G-20 min), LFA-1 has moved and is mainly associated with GM₁ and PI3K (directly or via X) that binds with GM₁, and GM₃ to a lesser extent. SHP-2 is detected in detergent-soluble domains where it only associates with PI3K. It is also detected in DRMs without association with gangliosides, p56^{lck} or PI3K.

sequester these proteins and consequently inhibit LFA-1-dependent adhesion.

We thank J. Lipeczka for invaluable help with image acquisition with the confocal microscope, and R. Fagard for the helpful discussions. M.T. was supported by a doctoral fellowship from La Ligue Nationale Contre le Cancer. This work was supported by Inserm.

REFERENCES

- Garofalo, T., Lenti, L., Longo, A., Misasi, R., Mattei, V., Pontieri, G. M., Pavan, A. and Sorice, M. (2002) Association of GM3 with Zap-70 induced by T cell activation in plasma membrane microdomains: GM3 as a marker of microdomains in human lymphocytes. *J. Biol. Chem.* **277**, 11233–11238.
- Mazerolles, F., Barbat, C., Trucy, M., Kolanus, W. and Fischer, A. (2002) Molecular events associated with CD4-mediated down-regulation of LFA-1-dependent adhesion. *J. Biol. Chem.* **277**, 1276–1283.
- Kniep, B., Cineke, T., Angelisova, P. and Horejsi, V. (1994) Association of the GPI-anchored leucocyte surface glycoproteins with ganglioside GM₃. *Biochem. Biophys. Res. Commun.* **203**, 1069–1075.
- London, E. and Brown, D. A. (2000) Insolubility of lipids in Triton X-100: physical origin and relationship to sphingolipid/cholesterol membrane domains (rafts). *Biochim. Biophys. Acta* **1508**, 182–195.
- Brown, D. A. and Rose, J. K. (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. *Cell* **68**, 533–544.
- Melkonian, K. A., Ostermeyer, A. G., Chen, J. Z., Roth, M. G. and Brown, D. A. (1999) Role of lipid modifications in targeting proteins to detergent-resistant membrane rafts. *J. Biol. Chem.* **274**, 3910–3917.
- Viola, A., Schroeder, S., Sakakibara, Y. and Lanzavecchia, A. (1999) T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* **283**, 680–682.
- Simons, K. and Toomre, D. (2000) Lipid rafts and signal transduction. *Nat. Rev. Mol. Cell Biol.* **1**, 31–41.
- Kabouridis, P. S., Janzen, J., Magee, A. and Ley, S. C. (2000) Cholesterol depletion disrupts lipid rafts and modulates the activity of multiple signaling pathways in T lymphocytes. *Eur. J. Immunol.* **30**, 954–963.
- Sorice, M., Parolini, I., Sansolini, T., Garofalo, T., Dolo, V., Sargiacomo, M., Tai, T., Peschle, C., Torrisi, M. R. and Pavan, A. (1997) Evidence for the existence of ganglioside-enriched plasma membrane domains in human peripheral lymphocytes. *J. Lipid Res.* **38**, 969–980.
- Parolini, I., Topa, S., Sorice, M., Pace, A., Ceddia, P., Montesoro, E., Pavan, A., Lisanti, M. P., Peschle, C. and Sargiacomo, M. (1999) Phorbol ester-induced disruption of the CD4-Lck complex occurs within a detergent-resistant microdomain of the plasma membrane. *J. Biol. Chem.* **274**, 14176–14187.
- Fragoso, R., Ren, D., Zhang, X., Wei-Chih Su, M., Burakoff, S. J. and Jin, Y. J. (2003) Lipid raft distribution of CD4 depends on its palmitoylation and association with Lck, and evidence for CD4-induced lipid raft aggregation as an additional mechanism to enhance CD3 signaling. *J. Immunol.* **170**, 913–921.
- Kabouridis, P. S., Magee, A. I. and Ley, S. C. (1997) S-acylation of Lck protein tyrosine kinase is essential for its signaling function in T lymphocytes. *EMBO J.* **16**, 4983–4998.
- Nguyen, D. H., Giri, B., Collins, G. and Taub, D. D. (2005) Dynamic reorganization of chemokine receptors, cholesterol, lipid rafts, and adhesion molecules to sites of CD4 engagement. *Exp. Cell Res.* **304**, 559–569.
- Leitinger, B. and Hogg, N. (2002) The involvement of lipid rafts in the regulation of integrin function. *J. Cell Sci.* **115**, 963–972.
- Pluskota, E., Chen, Y. and D'Souza, S. (2000) Src homology domain 2-containing tyrosine phosphatase 2 associates with intercellular adhesion molecule 1 to regulate cell survival. *J. Biol. Chem.* **275**, 30029–30036.
- Lacalle, R. A., Mira, E., Gomez-Mouton, C., Jimenez-Baranda, S., Martinez, A. C. and Manes, S. (2002) Specific SHP-2 partitioning in raft domains triggers integrin-mediated signaling via Rho activation. *J. Cell Biol.* **157**, 277–289.
- Xu, F., Zhao, R., Peng, Y., Guerrah, A. and Zhao, Z. J. (2001) Association of tyrosine phosphatase SHP-2 with F-actin at low cell densities. *J. Biol. Chem.* **276**, 29479–29484.

- 19 Inagaki, K., Nogushi, T., Matosaki, T., Horikawa, T., Fukunaga, K., Tsuda, M., Ichihashi, M. and Kasuga, M. (2000) Roles for the protein tyrosine phosphatase SHP-2 in cytoskeletal organization, cell adhesion and cell migration revealed by overexpression of a dominant negative mutant. *Oncogene* **19**, 75–84
- 20 Munoz, P., Navarro, M., Pavon, E., Salmeron, J., Malavasi, F., Sancho, J. and Zubiaur, M. (2003) CD38 signaling in T cells is initiated within a subset of membrane rafts containing Lck and the CD3 ζ subunit of the T cell antigen receptor. *J. Biol. Chem.* **278**, 50791–50802
- 21 Giagulli, C., Scarpini, E., Ottoboni, L., Narumiya, S., Butcher, E. C., Constantin, G. and Laudanna, C. (2004) RhoA and ζ PKC control distinct modalities of LFA-1 activation by chemokines: critical role of LFA-1 affinity triggering in lymphocytes *in vivo* homing. *Immunity* **20**, 25–35
- 22 Nagel, W., Zeitlmann, L., Schilcher, P., Geiger, C., Kolanus, J. and Kolanus, W. (1998) Phosphoinositide 3-OH kinase activates the β 2 integrin adhesion pathway and induces membrane recruitment of cytohesin-1. *J. Biol. Chem.* **273**, 14853–14861
- 23 Constantin, G., Majeed, M., Giagulli, C., Piccio, L., Kim, J. Y., Butcher, E. C. and Laudanna, C. (2000) Chemokines trigger immediate β 2 integrin affinity and mobility changes: differential regulation and roles in lymphocyte arrest under flow. *Immunity* **13**, 759–769
- 24 Trucy, M., Barbat, C., Sorice, M., Fischer, A. and Mazerolles, F. (2004) CD4-induced down-regulation of T cell adhesion to B cells is associated with localization of phosphatidylinositol 3-kinase and LFA-1 in distinct membrane domains. *Eur. J. Immunol.* **34**, 2168–2178
- 25 Mazerolles, F., Barbat, C., Meloche, S., Graton, S., Soula, M., Fagard, R., Fischer, S., Hivroz, C., Bernier, J., Sekaly, R. P. and Fischer, A. (1994) LFA-1-mediated antigen-independent T cell adhesion is regulated by CD4 and p56^{lck} tyrosine kinase. *J. Immunol.* **152**, 5670–5679
- 26 Smart, E. J., Ying, Y. S., Mineo, C. and Anderson, R. G. W. (1995) A detergent-free method for purifying caveolae membrane from tissue culture cells. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 10104–10108
- 27 Katagiri, Y. U., Kiyokawa, N. and Fujimoto, J. (2001) A role for lipid rafts in immune cell signaling. *Microbiol. Immunol.* **45**, 1–8
- 28 Mazerolles, F., Amblard, F., Lumbroso, C., Lecomte, O., Van De Moortele, P., Barbat, C., Piatier-Tonneau, D., Auffray, C. and Fischer, A. (1990) Regulation of T helper-B lymphocyte adhesion through CD4–HLA class II interaction. *Eur. J. Immunol.* **20**, 637–644
- 29 Mazerolles, F., Barbat, C., Hivroz, C. and Fischer, A. (1996b) Phosphatidylinositol 3-kinase participates in p56^{lck}/CD4 dependent down-regulation of LFA-1 mediated T cell adhesion. *J. Immunol.* **157**, 4844–4854.
- 30 Hivroz, C., Mazerolles, F., Soula, M., Fagard, R., Graton, S., Meloche, S., Sekaly, R. P. and Fischer, A. (1993) Human immunodeficiency virus (HIV) glycoprotein 120 and derived peptides activate protein tyrosine kinase in human CD4 T lymphocyte. *Eur. J. Immunol.* **23**, 600–607
- 31 Saggiaro, D., Sorio, C., Calderazzo, F., Callegaro, L., Panozzo, M., Berton, G. and Chieco-Bianchi, L. (1993) Mechanism of action of the monosialoganglioside G_{M1} as a modulator of CD4 expression: evidence that G_{M1}–CD4 interaction triggers dissociation of p56^{lck} from CD4, and CD4 internalization and degradation. *J. Biol. Chem.* **268**, 1368–1375
- 32 Sorice, M., Garofalo, T., Misasi, R., Longo, A., Mikulak, J., Dolo, V., Pontieri, G. M. and Pavan, A. (2000) Association between G_{M3} and CD4–Lck complex in human peripheral blood lymphocytes. *Glycoconj. J.* **17**, 247–252
- 33 Ilangumaran, S., Arni, S., Van Echten-Deckert, G., Borisch, B. and Hoessli, D. (1999) Microdomain-dependent regulation of Lck and Fyn protein-tyrosine kinases in T lymphocyte plasma membranes. *Mol. Biol. Cell* **10**, 891–905
- 34 Dykstra, M., Cherukuri, A., Sohn, H. W., Tzeng, S. J. and Pierce, S. K. (2003) Location is everything: lipid rafts and immune cell signaling. *Annu. Rev. Immunol.* **21**, 457–481
- 35 Manes, S., Lacalle, R. A., Gomez-Mouton, C. and Martinez, A. C. (2003) From rafts to crafts: membrane asymmetry in moving cells. *Trends Immunol.* **24**, 319–325
- 36 Mitchell, C. A., Kanca, O. and McIntyre, B. W. (2002) Lipid microdomain clustering induces a redistribution of antigen recognition and adhesion molecules on human T lymphocytes. *J. Immunol.* **168**, 2737–2744
- 37 Garofalo, T., Sorice, M., Misasi, R., Cinque, B., Giammatteo, M., Pontieri, G. M., Cifone, M. G. and Pavan, A. (1998) A novel mechanism of CD4 down-modulation induced by monosialoganglioside G_{M3}: involvement of serine phosphorylation and protein kinase C δ translocation. *J. Biol. Chem.* **273**, 35153–35160
- 38 Iwabuchi, K., Yamamura, S., Prinetti, A., Handa, K. and Hakomori, S. (1998) G_{M3}-enriched microdomain involved in cell adhesion and signal transduction through carbohydrate–carbohydrate interaction in mouse melanoma B16 cells. *J. Biol. Chem.* **273**, 9130–9138
- 39 Zhang, S., Yang, W., Kontaradis, M., Bivona, T., Wen, G., Araki, T., Luo, J., Thompson, J., Schraven, B., Philips, M. and Neel, B. G. (2004) Shp2 regulates SRC family kinase activity and Ras/Erk activation by controlling Csk recruitment. *Mol. Cell* **13**, 341–355

Received 13 July 2006/14 November 2006; accepted 24 November 2006

Published as BJ Immediate Publication 24 November 2006, doi:10.1042/BJ20061061