Extensive temporally regulated reorganization of the lipid raft proteome following T-cell antigen receptor triggering

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Signalling by immunoreceptors is orchestrated at specific plasma membrane microdomains, referred to as lipid rafts. Here we present a proteomics approach to the temporal analysis of protein association with lipid rafts following T-cell antigen receptor (TCR) triggering. We show that TCR engagement promotes the temporally regulated recruitment of proteins participating in the TCR signalling cascade to lipid rafts. Furthermore, TCR triggering results in profound modifications in the composition of lipid rafts involving a number of proteins associated either directly or indirectly with both plasma and intracellular membranes. Raft-associated proteins can be clustered according to their temporal profile of raft association. The data identify lipid rafts as highly dynamic structures and reveal a dramatic impact of surface TCR triggering not only on components of the TCR signalling machinery but also on proteins implicated in a number of diverse cellular processes.

Key words: membrane microdomain, protein segregation, proteomics, signal transduction, T-lymphocyte.

INTRODUCTION

T-cell commitment to proliferation and subsequent differentiation is the endpoint of a complex signalling cascade initiated in response to T-cell antigen receptor (TCR) engagement and leading to activation of gene expression [1]. A number of additional and diverse cellular processes, which contribute either to the topological organization of the immunological synapse or to signal extinction, are concomitantly initiated, including reorganization of cortical actin [2], TCR down-regulation by endocytosis [3] and export to the cell surface by exocytosis of the negative regulator cytotoxic T-lymphocyte antigen-4 (CTLA-4) [4]. Hence lateral mobility of plasma-membrane-associated cell-surface molecules, as well as membrane trafficking, are essential elements in the control of T-cell activation. Furthermore, emerging evidence underlines the requirement for compartmentalization of the signalling cascade to specific membrane domains, known as lipid rafts, for productive TCR signalling [5].

Lipid rafts are viewed as dynamic assemblies of cholesterol and glycosphingolipids which, as the result of their biophysical and structural properties, generate microdomains characterized by a liquid-ordered state within the liquid-disordered glycerophospholipid membrane bilayer [6]. The tightly packed lipid environment of lipid rafts favours their interactions with the saturated acyl chains of glycosylphosphatidylinositol (GPI)-anchored proteins [7]. The same features dictate association with lipid rafts of acylated cytosolic and transmembrane proteins, including Src kinases, small and heterotrimeric G-proteins, the CD4 and CD8 co-receptors and the linker of activated T-cells (LAT) adaptor [5]. Whereas the actual existence of sphingolipid/cholesterol-enriched microdomains in cellular membranes awaits formal proof [7], their distintive biochemical features, i.e. relative resistance to solubilization by non-ionic detergents and buoyancy on density gradients, as well as the availability of tools such as sterol-binding drugs, have provided an operational method to assess the role of lipid rafts in biomembranes [8].

Initially implicated in the sorting of GPI-anchored proteins to the apical membrane of polarized cells, lipid rafts are now believed to play a wider role in the major routes of membrane trafficking, including transport of GPI-anchored proteins and glycosphingolipids to the cell surface, regulated secretion, transport from the endosomes to the Golgi apparatus and internalization via both caveolae and chlathrin-coated pits [8,9]. Furthermore, recent work has highlighted a strategic role for lipid rafts in immunoreceptor signalling. In T-cells key components of the TCR signalling machinery, such as Src kinases and LAT, are segregated to lipid rafts, while the TCR itself inducibly associates with lipid rafts following encounter with antigen [10–12]. This translates into a two-component machinery which ensures full activation of the signalling cascade only in the presence of the appropriate trigger, while providing a pre-existing high concentration of the molecules required for phosphorylation of the CD3/ζ subunits and assembly of the signalling complex. In addition, the preferential association with lipid rafts of CD4/CD8 and GPI-anchored co-stimulatory receptors is likely to stabilize the contact surface between T-cell and antigen-presenting cell and thereby enhance signalling both directly and indirectly. In agreement with their role as signalling platforms, the composition in signalling proteins of rafts from activated cells closely resembles that of the immunological synapse [13].

Because of their involvement in multiple processes triggered by the TCR, looking at the dynamics of protein association with lipid rafts in response to TCR engagement is likely to provide an insight into the timing and extent of propagation of the signal activated by an individual cell-surface receptor. Here we present

Abbreviations used: ARF, ADP ribosylation factor; 1D, one-dimensional; 2D, two-dimensional; ERK, extracellular signal-regulated protein kinase; GM130, Golgi matrix protein 130; GPI, glycosylphosphatidylinositol; LAT, linker of activated T-cells; MALDI-TOF, matrix-assisted laser-desorption ionization–time-of-flight; PLC, phospholipase C; SLP-76, SH2-domain-containing leucocyte protein of 76 kDa; TCR, T-cell antigen receptor; VDAC, voltage-dependent anion channel; ZAP-70, zeta-associated protein of 70 kDa.

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a functional proteomics approach to the analysis of the temporal changes in protein composition induced in lipid rafts by TCR triggering. The results show that rafts are highly dynamic structures where clusters of proteins with similar temporal patterns of raft association can be tracked. Identification of a number of the major proteins reveals a profound impact of TCR occupancy not only on components of the TCR-associated signalling cascade but also on a number of proteins implicated in diverse cellular processes.

EXPERIMENTAL

Antibodies

Polyclonal rabbit antisera against phospholipase C (PLC) γ, LAT, Grb2, Rab5, extracellular signal-regulated protein kinase (ERK) and p38 were purchased from Upstate Biotechnology (Boston, MA, U.S.A.) and Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal rabbit antibodies against a glutathione S-transferase-CD3δ fusion protein (C. Ulivieri and C. T. Baldari, unpublished work) were raised as described previously [14]. Monoclonal antibodies against zeta-associated protein of 70 kDa (ZAP-70), Lck, Fyn, Shc, Vav, Ras, Bcl-2, cathepsin D, CD3ζ, CD3ϵ and phosphotyrosine were purchased from Upstate Biotechnology, Santa Cruz Biotechnology, Transduction Laboratories (Lexington, KY, U.S.A.) and Calbiochem Novabiochem GmbH (Bad Soden, Germany). A sheep polyclonal anti-SH2-domain-containing leucocyte protein of 76 kDa (SLP-76) antiserum was kindly provided by Dr G. Koretzky (Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, U.S.A.). Antibodies against the Golgi markers p115, ADP ribosylation factor (ARF) and Golgi matrix protein 130 (GM130) were generous gifts of Dr A. De Matteis [Department of Cell Biology and Oncology, Consorzio Mario Negri Sud, S. Maria Imbaro (Chieti), Italy]. Anti-actin, anti-tubulin and peroxidase-conjugated secondary antibodies were purchased from Amersham Biosciences Italia (Milan, Italy). IgG from OKT3 hybridoma (ATCC; CRL 8001) supernatants were purified on Mabtrap (Amersham Biosciences Italia) and titrated by flow cytometry.

Cell activations and purification of lipid rafts

Activations were carried out as described in [15] by TCR cross-linking using OKT3 and anti-mouse antibodies. Saturating concentrations of OKT3 (as determined by flow cytometry) were used. After a 30 min incubation on ice, unbound antibody was washed and bound OKT3 was cross-linked with 50 μg/ml secondary antibody. The ratio of antibodies to cell equivalents was kept constant. Lipid rafts were purified from 50×10^6 cells/sample by density-gradient centrifugation as described in [16] using Brij 58 as a detergent. Alternatively, 500×10^6 cells were used for the preparative two-dimensional (2D) gel. A fixed ratio of detergent to cell equivalents (1 mg/10^7 cells) was used in all raft preparations. Equal amounts of proteins from raft and non-raft fractions, quantificated using a kit from Pierce (Rockford, IL, U.S.A.), were separated by SDS/PAGE. To normalize for the significantly lower amounts of proteins recovered in the low-density fractions as compared with high-density fractions, equal amount of proteins were loaded. These corresponded to $\approx 40 \times 10^6$ cell equivalents for low-density fractions and $\approx 2 \times 10^6$ cell equivalents for high-density fractions. For the 2D gel analysis lipid raft-enriched samples were resuspended in 8 M urea, 4 % (w/v) CHAPS and 1 % (w/v) dithioerythritol after the concentration step. Disruption of rafts was achieved by incubating the cells in serum-free medium for 30 min at 37 °C in the presence of 15 mM methyl-β-cyclodextrin (Sigma Italia srl, Milan, Italy) prior to cell lysis.

2D gel electrophoresis

2D electrophoresis was performed using the Immobiline-polyacrylamide system as described in [17]. Isoelectric focusing was carried out on non-linear wide-range of immobilized pH gradients (pH 3–10; 18 cm-long immobilized pH gradient strips) using the IPGphor system (Amersham Biosciences, Uppsala, Sweden). The second dimension was carried out on 9–16 % polyacrylamide linear gradient gels (18 cm $\times$ 20 cm $\times$ 1.5 mm) at 40 mA/gel constant current and 10 °C until the dye front reached the bottom of the gel. Analytical gels were stained with ammoniacal silver nitrate. Preparative gels for mass spectrometric analysis were stained with 0.2 % (w/v) Coomassie Blue R250 in 50 % (v/v) methanol and 10 % (v/v) acetic acid and destained in 50 % methanol. Silver- and Coomassie Blue-stained gel images were digitized using a Molecular Dynamics 300S laser densitometer. Computer-aided 2D image analysis was carried out using MELANIE 3 software (GeneBio, Geneva, Switzerland).

Protein identification by Western blotting

After one-dimensional (1D) and 2D electrophoresis proteins were electroblotted on to nitrocellulose membrane. Before 2D immunodetection the membranes were reversibly stained with 0.2 % (w/v) Ponceau S in 3 % (w/v) trichloroacetic acid for 3 min [18] to facilitate the further computer-aided matching of immunoreactive spots on the silver- and Coomassie Blue-stained protein maps. Immunoreactive spots were detected using an enhanced chemiluminescence (ECL) detection kit (Pierce) and peroxidase-conjugated secondary antibodies. Stripping was carried out by washing the filters three times for 45 min in 0.1 M glycine, pH 2.8, followed by two 20 min washes in Tris-buffered saline with 0.02 % Tween 20. The procedure was repeated, and filters were subsequently saturated in 3 % non-fat milk in Tris-buffered saline with 0.02 % Tween 20 before reprobing. The effectiveness of the stripping procedure was routinely checked by immunoblotting stripped filters with secondary antibody alone. ECL images and silver-stained reference maps were compared using MELANIE 3 software.

Protein identification by matrix-assisted laser-desorption ionization–time-of-flight (MALDI-TOF) MS

2D-separated spots were identified by MALDI-TOF peptide mass fingerprinting as described previously [19]. After visualization with Coomassie Blue staining, electrophoretic spots were excised, destained and dehydrated with acetonitrile for subsequent rehydration with trypsin solution. Tryptic digestion was carried out overnight at 37 °C. Each protein digest (0.75 μl) was spotted on to the MALDI instrument target and allowed to dry. Then 0.75 μl of matrix solution [saturated solution of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 0.5 % (v/v) trifluoroacetic acid] was applied to the dried sample and dried again. Mass spectra were obtained using an ETTAN MALDI-TOF mass spectrometer from Amersham Biosciences (Uppsala, Sweden). Database searching was carried out using the ProFound algorithm (http://129.85.19.192/profound_bin/WebProFound.exe) searching in the NCBInr database. Unambiguous protein identification was achieved for 29 2D-separated protein spots, with an average of eight peptide masses matched per protein as limited by the following searching criteria: less than 150 p.p.m. difference in mass between

experimental and theoretical peptide masses, more than 20% of protein sequence coverage required and a Z score higher than 1.6. For all identified proteins the probability value was the maximum possible (i.e. 1.0e+000).

Cluster analysis

Cluster analysis was carried out using the EPCLUST tool for clustering and analysis of gene-expression data within Expression Profiler, a set of tools for microarray analysis developed by Jaak Vilo at the European Bioinformatics Institute [20], available online at the following web site: http://ep.ebi.ac.uk/. The data were clustered and visualized as hierarchical clustering using Pearson linear correlation-based distance (centred).

RESULTS AND DISCUSSION

Temporally regulated recruitment of signalling proteins to lipid rafts in response to TCR triggering

Lipid rafts were purified by sucrose gradient centrifugation from lysates of Jurkat T-cells. Immunoblot analysis of light fractions, containing the buoyant detergent-insoluble membranes, and heavy fractions, enriched in detergent-soluble proteins, showed that, in the absence of TCR triggering, the raft-resident proteins Lck, Fyn, LAT and Ras were segregated to the light fractions, whereas cytosolic proteins participating in the TCR signalling cascade, such as ZAP-70, Shc, SLP-76, Vav, PLCγ and the mitogen-activated kinases ERK and p38, were mostly recovered in the heavy fractions (Figure 1), confirming the enrichment in lipid rafts of the light fractions. Interestingly, significant levels of Grb2 were found in the detergent-insoluble fractions in the absence of receptor stimulation (Figure 1A). Immunoblot analysis of the same samples with antibodies against CD3ζ, CD3δ and CD3ϵ confirmed a weak association of the unstimulated TCR–CD3 complex with lipid rafts (Figure 1A).

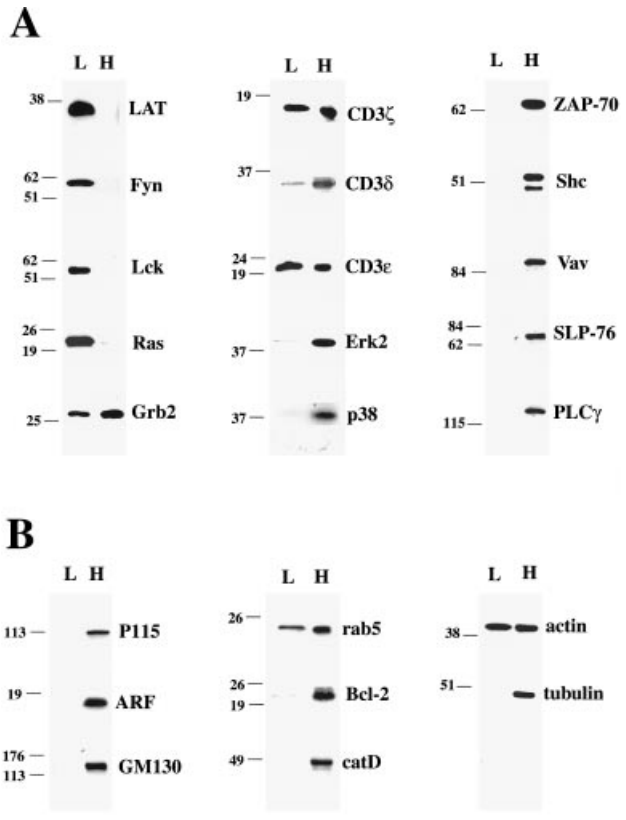

Figure 1 Characterization of purified lipid rafts

(**A**) Components of the TCR–CD3 complex and associated signalling cascade; (**B**) organelle markers and cytoskeletal proteins. Immunoblot analysis was performed on detergent-resistant (L, light fractions) and detergent-soluble (H, heavy fractions) proteins from unstimulated Jurkat cells. Equal amounts of protein were loaded into each lane. The filter was sequentially probed with the antibodies indicated. The migration of molecular-mass markers is indicated.

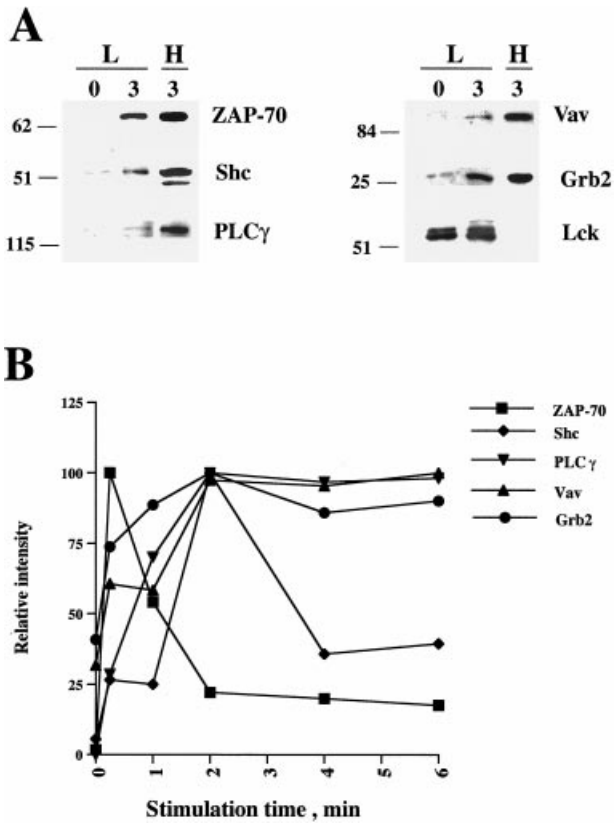

Figure 2 Temporally regulated recruitment of signalling proteins to lipid rafts in response to TCR triggering

(**A**) Immunoblot analysis with anti-ZAP-70, anti-PLCγ, anti-Vav, anti-Shc, anti-Grb2 and anti-Lck antibodies of detergent-resistant (L, light fractions) and detergent-soluble (H, heavy fractions) proteins from Jurkat cells either unstimulated (0) or activated by TCR/CD3 cross-linking for 15 s (3). Equal amounts of protein were loaded into each lane. The filter was sequentially probed with the indicated antibodies. The migration of molecular-mass markers is indicated. (**B**) Quantification by laser densitometry of the bands immunoreactive for ZAP-70, PLCγ, Vav, Shc and Grb2 in lipid rafts purified from Jurkat cells either unstimulated or activated by TCR/CD3 cross-linking for different times as indicated. Equal amounts of protein were loaded into each lane. Values were normalized to the levels of Lck in each lane, as determined by immunoblot. The results of a representative experiment are shown.

Contamination of lipid rafts by abundant detergent-soluble proteins was ruled out by probing purified rafts with anti-tubulin antibodies. As shown in Figure 1(B), tubulin co-purified solely with non-raft proteins, as opposed to actin, which has been previously shown to be present as F-actin in this location [21]. Furthermore, neither the Golgi markers GM130, p115 and ARF, nor the lysosome marker cathepsin D or the mitochondrial protein Bcl-2, co-purified with lipid rafts, while the early endosome marker Rab5 partially segregated to these fractions, possibly as the result of its post-translational addition with a prenyl group. Of note, these markers include soluble proteins

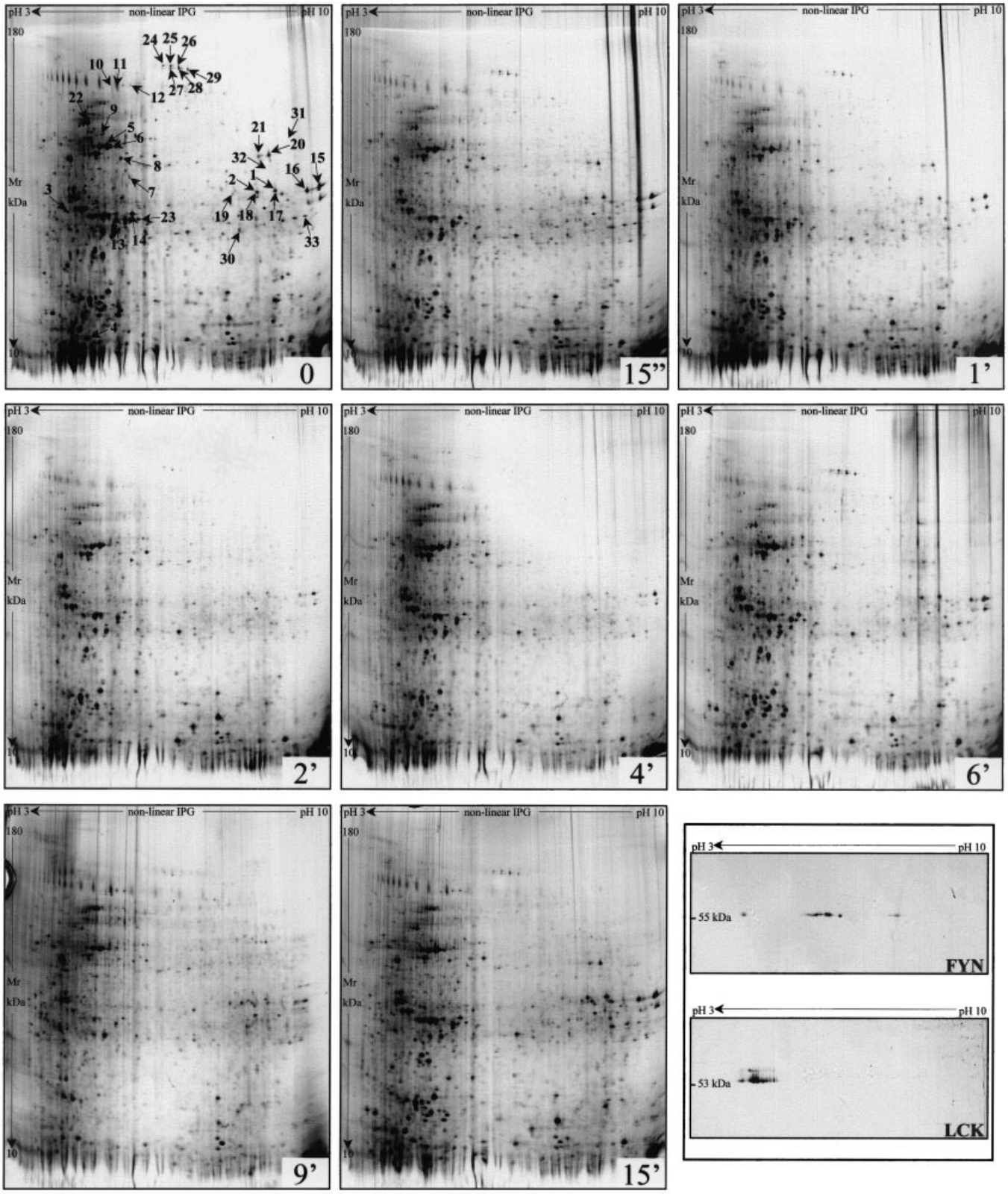

Figure 3 Global analysis of the dynamics of protein recruitment to lipid rafts induced by TCR triggering

Silver staining of the proteins enriched in lipid rafts purified from Jurkat cells, either unstimulated or activated for different times by anti-CD3 monoclonal antibody cross-linking, and resolved by 2D-gel electrophoresis. The migration of molecular-mass markers and the pI gradient are indicated. The numbers in the panel corresponding to time 0 refer to the protein spots identified, which are listed in Table 2. The boxes in the lower right refer to an immunoblot analysis of lipid rafts from resting Jurkat cells using anti-Lck and anti-Fyn monoclonal antibodies. IPG, immobilized pH gradient.

(cathepsin D, GM130 and ARF), membrane-bound proteins (Rab5) and transmembrane proteins (p115 and Bcl-2), ruling out a preferential co-purification with lipid rafts of membrane proteins and confirming the purity of the lipid raft preparations used in this study.

As shown in Figure 2(A), ZAP-70, PLCγ, Vav and Shc were inducibly and rapidly recruited to lipid rafts following TCR engagement, with a concomitant increase in the levels of raft-associated Grb2. Of note, only a small fraction of the total cellular complement of these proteins co-purified with lipid rafts (Figure 2A). No significant variation in the levels of the raft resident proteins Lck, Fyn and LAT was observed following stimulation (Figure 2A and results not shown). To follow the dynamics of recruitment to lipid rafts of proteins implicated in the TCR signalling cascade, the time course of raft association of ZAP-70, PLCγ, Vav, Shc and Grb2 was determined by immunoblot analysis of rafts purified from Jurkat cells activated by TCR cross-linking for 15 s and 1, 2, 4 and 6 min. As shown in Figure 2(B), the kinetics of recruitment of each protein were not overlapping. The levels of ZAP-70 recruitment to lipid rafts peaked at 15 s and dropped rapidly, suggesting a transient requirement for ZAP-70 in the early phase of the raft-associated signalling cascade, marked by the assembly of a multimolecular complex on LAT [22]. The association of Shc with lipid rafts was delayed as compared with ZAP-70, in agreement with a requirement for ZAP-70 for stable inclusion of Shc into the TCR-associated signalling complex [14]. This association was significantly decreased by 4 min.

As opposed to the transient association of ZAP-70 and Shc with lipid rafts, the association of Vav, PLCγ and Grb2 was sustained, after reaching a plateau at 2 min (Figure 2B). Of note, the levels of PLCγ increased steadily up to 2 min, while Vav and Grb2 were immediately up-regulated in response to TCR triggering, in agreement with a later recruitment of PLCγ to the signalling complex following LAT phosphorylation by ZAP-70 [23]. Hence proteins participating in the TCR signalling cascade associate with lipid rafts in a temporally regulated fashion which parallels their recruitment to the signalling complex [24], supporting a key role for raft localization in the inducible assembly of this complex.

TCR triggering induces a rapid and massive reorganization of lipid rafts

To get an insight into the dynamics of lipid rafts induced by TCR engagement, a global analysis of raft-associated proteins was undertaken. Lipid rafts were purified by sucrose gradient centrifugation from Jurkat cells either unstimulated or activated for 15 s and 1, 2, 4, 6, 9 and 15 min by TCR cross-linking. Proteins recovered from raft-enriched fractions were resolved by 2D gel electrophoresis and visualized by silver staining. As shown in Figure 3, the protein composition of lipid rafts from resting cells appears quite complex, with $\approx$ 900 reproducibly detectable spots, as revealed by a computer-generated synthetic gel which included all spots common to three individual 2D gels of raft samples. Of note, the large majority of these spots were either completely absent or dramatically reduced in rafts purified from cells where lipid rafts had been disrupted with methyl-β-cyclodextrin, a cholesterol-binding drug (results not shown), supporting the identity of these spots as raft-associated proteins. TCR triggering resulted in a rapid and dramatic reorganization of lipid rafts, as revealed by the extensive differences in the protein pattern of rafts purified from activated cells at the different time points (Figure 3). These differences were reproducibly detectable in different time-course experiments (results not shown).

Table 1 Analysis of the data presented in Figure 3

All detectable spots in the silver-stained gels were quantificated by laser densitometry and normalized to the total protein content in each gel. The intensity of each spot in the samples from the different time points was converted to a relative value, expressed as a percentage of the maximum value for each specific spot (set at 100%).

Total number of spots	872
Number of spots with maximal intensity at each time point	
0	458 (52.6%)
15 s	32 (3.7%)
1 min	8 (0.9%)
2 min	49 (5.6%)
4 min	39 (4.5%)
6 min	34 (3.9%)
9 min	178 (29.4%)
15 min	44 (5.1%)
Number of spots undetectable at one or more time points	551 (63.3%)
Number of spots with maximal intensity at more than one time point	30 (3.4%)
Number of spots with $\geqslant$ 3-fold variation between time points	764 (87.6%)
Number of spots with $\leqslant$ 2.5-fold variation between time points	43 (4.9%)

The intensity of all visible spots was determined by laser densitometry and normalized to the total protein content of the sample. The relative intensity of individual spots at the different time points was then calculated as a percentage of the maximal intensity, set for each spot at 100%. As shown in Table 1, $\approx$ 800 out of the $\approx$ 900 reproducible spots varied by $\geqslant$ 3-fold at some time point, supporting an extensive reorganization of lipid rafts in response to TCR triggering. Furthermore, for each time point a subset of the total number of spots exhibited maximal intensity, demonstrating a temporal regulation of lipid raft reorganization, characterized by sequential waves of maximal association of subsets of proteins with lipid rafts. Of note, only $\approx$ 500 proteins were completely undetectable at one or more of the time points, suggesting that the variation in protein composition of lipid rafts is in large part quantitative, in agreement with the concept of the lipid raft as a liquid-ordered phase where specific membrane-associated components preferentially segregate [6]. About 50% of the spots showed maximal intensity in rafts from unstimulated cells, implying a massive and rapid protein exclusion from lipid rafts following TCR engagement. A subset of 43 spots harbour limited temporal variation ($\leqslant$ 2.5-fold variation) and are likely to represent stable lipid raft residents.

Cluster analysis of the temporal variations in lipid raft proteins

In addition to their time of maximal association with lipid rafts, proteins co-purifying with rafts showed a wide variation in their temporal pattern of association, measured as the relative intensity of the respective spots at different time points. To highlight proteins with similar patterns of temporal variation we performed a cluster analysis based on the algorithms for the analysis of DNA microarray expression data [20]. The results are presented in Figure 4(A), which shows both the variations in relative intensity of each spot plotted against time, and the relationships among the patterns of temporal variation. Seven major clusters were identified. The large clusters 2 and 6 were further subdivided into two and three subclusters, respectively. The average profile of the relative intensities of the spots in each cluster plotted against time, as well as the number of spots in each cluster, are shown in Figure 4(B).

Clusters 3, 4, 5 and 7 include proteins whose level of association with lipid rafts increases in response to TCR engagement, albeit with different kinetics, the time of maximal association

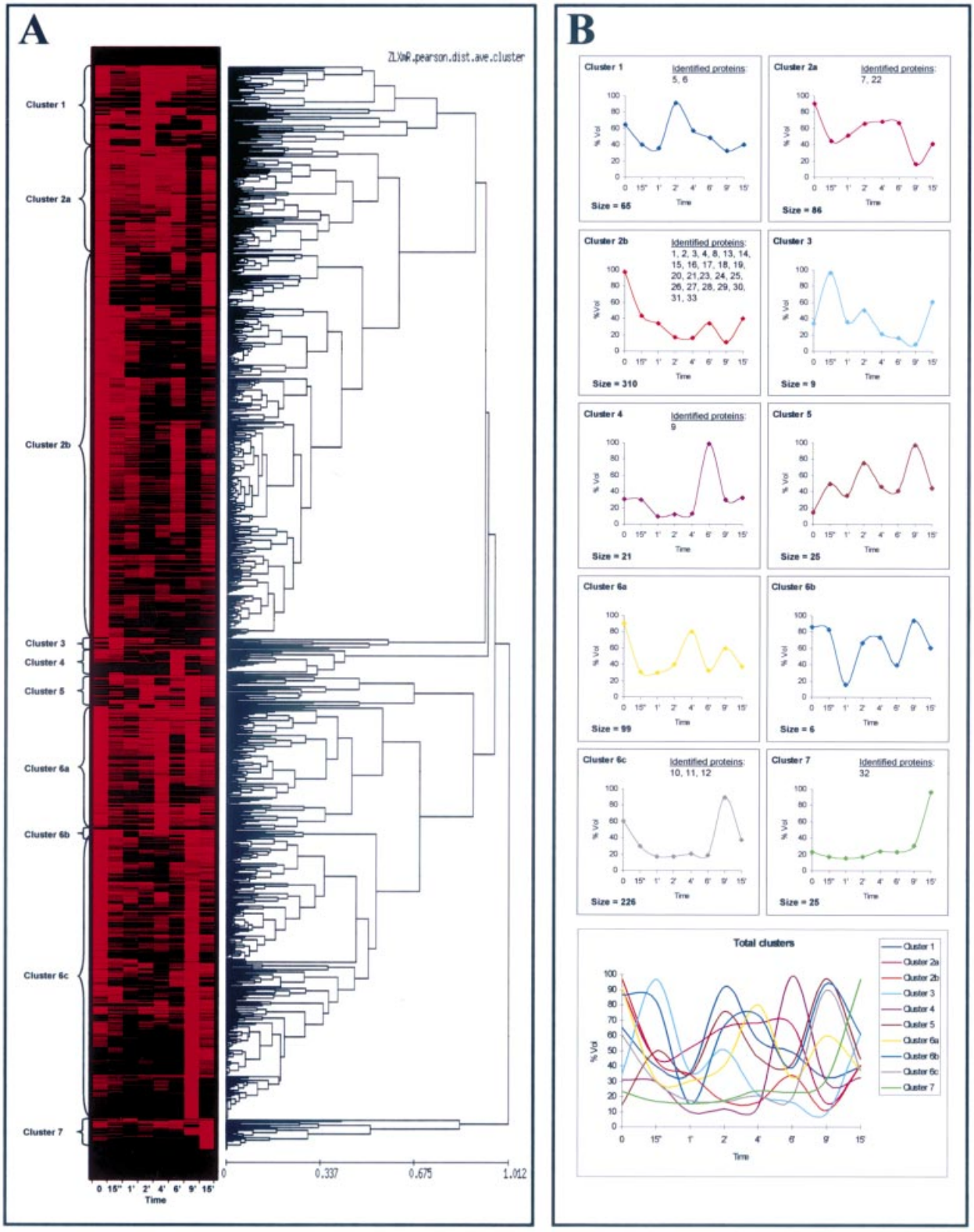

Figure 4 Cluster analysis of the dynamics of protein recruitment to lipid rafts induced by TCR triggering

(**A**) Left-hand side: relative intensity of each spot revealed by silver staining in lipid rafts purified from Jurkat cells activated for different times by anti-CD3 monoclonal antibody cross-linking, and resolved by 2D-gel electrophoresis. Spots are ordered from top to bottom, time (unstimulated, or activated for 15 s, 1, 2, 4, 6, 9 and 15 min) from left to right. The relative intensity (% vol) of each spot is proportional to the intensity of the colour. Right-hand side: average linkage clustering based on Pearson linear correlation distance. The major clusters are numbered from top to bottom. (**B**) Average profiles of temporal variations for each cluster of proteins shown in (**A**) and overlap of the average profiles of all clusters (bottom panel). The total number of spots/cluster, as well as the number assigned to each of the spots identified in each cluster, are indicated (see Table 2 for the identity of the spots).

being respectively 15 s, 6 min, 9 min and 15 min for clusters 3, 4, 5 and 7. Conversely, clusters 1, 2 and 6 are represented by proteins whose level of association with lipid rafts decreases following TCR triggering. After the initial drop in the levels of raft association, these proteins show a general trend to reassociate with lipid rafts, again with different kinetics. By 15 min the proteins of most clusters have returned to their initial level of raft association detected in the absence of stimulation, suggesting that, albeit extensive, the changes in raft composition induced by TCR triggering are transient.

Although the extent of protein recruitment to or exclusion from lipid rafts might represent an overestimate, depending on a number of factors such as limited experimental variation, normalization to the protein content of the sample and the low range of linearity in protein quantification by silver staining, the results show that a complex and dynamic pattern of protein association with lipid rafts is triggered by the TCR.

Identification of major raft-associated proteins

2D gels of raft-enriched fractions from cells activated for 2 min by TCR cross-linking were analysed by immunoblot with anti-Lck, anti-Fyn, anti-LAT, anti-ZAP-70 and anti-Shc antibodies. The intensity of the immunoreactive spots on the 2D gels was significantly lower than the intensity of the respective immunoreactive bands on the 1D gels. Accordingly, only the raft-resident proteins Lck and Fyn were detectable (Figure 3 and results not shown). Lck was resolved as two isoelectric series with relative mobilities of 56 and 59 kDa, corresponding to the major immunoreactive species detected by anti-phosphotyrosine antibodies (results not shown), while Fyn was detectable as a single 59 kDa isoelectric series (Figure 3). Neither Lck nor Fyn could be matched to any of the silver-stained spots, indicating that, although they are segregated to lipid rafts, their relative abundance as compared with other raft-associated proteins is low. Like many transmembrane proteins, which fail to enter the isoelectrofocusing gel, LAT was undetectable by immunoblot in these conditions. In agreement with the inducible association with lipid rafts of only a minor part of the total cellular complement of cytosolic signalling proteins (Figure 2A), neither ZAP-70 nor Shc were detected (results not shown).

To identify GPI-anchored proteins, which are known to segregate to lipid rafts in the Golgi complex [25], Jurkat T-cells were metabolically labelled with [^{14}C]ethanolamine, the precursor of GPI anchors. Proteins recovered from raft-enriched fractions were separated by 2D gel electrophoresis and the spots corresponding to the labelled proteins were revealed by Phosphorimager analysis. Four spots were labelled at detectable levels, all of which could be matched to silver-stained spots (Figure 3). Two of the ^{14}C-labelled spots (1 and 2 in Table 2) overlapped with spots 17 and 18 respectively. The latter corresponded to two of the major spots detected by Coomassie Blue staining and were subsequently identified as voltage-dependent anion channel (VDAC) 2 isoforms by MS (Table 2). Since VDAC2 is a membrane porin, the ^{14}C-labelled spots 1 and 2 are likely to correspond to minor spots co-migrating with the major VDAC2 components; however, this point awaits further investigation.

Matching with public proteomics databases, as well as subsequent mass spectrometric analysis, permitted identification of actin, in agreement with a role for the cortical actin cytoskeleton in regulating the lateral mobility of rafts and thereby increasing their stability [21]. Interestingly, a transient up-regulation in the levels of raft-associated actin was observed (Figure 4B), in support of our finding of a raft-dependent phase of F-actin reorganization following TCR triggering [26]. A local accumulation of F-actin in raft patches formed by the GPI-anchored protein CD59 and the ganglioside GM1 has also been reported [27].

We analysed 29 major spots revealed by Coomassie Blue staining of a 2D-gel separation of lipid rafts by MS and identified 17 proteins present in public databases, seven of which corresponded to multiple spots with different pI values. Collectively, 23 proteins were identified. The results are summarized in Figure 3 and Table 2. Strikingly, none of the proteins identified by MS corresponds to any of the proteins known to participate in the TCR signalling cascade, including raft residents such as Lck and Fyn, supporting the notion that these proteins are minor components of the total protein complement of cellular rafts.

Of the proteins identified by MS, two correspond to hypothetical proteins encoded by human cDNA clones. All the remaining proteins have been described as associated, either directly or indirectly, with biomembranes (Table 2). Two proteins, the Gβ regulatory subunit of heterotrimeric G-proteins and stomatin-like 2, are associated with the plasma membrane as the result of the post-translational addition of a lipid moiety, and are known to segregate to lipid rafts [28,29]. TIP47 (tail-interacting protein of 47 kDa), a protein implicated in transport of the mannose 6-phosphate receptor from endosomes to the *trans*-Golgi network [30], and the molecular chaperones heat-shock cognate protein (HSC) 70 [31] and glucose-regulated protein (GRP) 75 [32], interact physically, albeit indirectly, and functionally with intracellular membranes, where they might segregate at least in part to lipid rafts. Finally, a significant number of proteins are associated with the outer [VDAC1, VDAC2, translocase of the outer mitochondrial membrane (TOM) 40, ATP synthase β] or inner (prohibitin, mitofilin, NADH dehydrogenase, ubiquinol-cytochrome *c* reductase) mitochondrial membranes [33–40]. In agreement with the lack of contamination of purified lipid rafts by abundant detergent-soluble proteins (Figure 1), no protein from the mitochondrial matrix was found among the major spots subjected to MS analysis (Table 2). Notwithstanding some differences possibly related to the detergent used for solubilization (Triton X-100 versus Brij-97), the type of electrophoretic separation (1D versus 2D gels) and the method used for protein identification (microcapillary liquid chromatography electrospray ionization tandem MS versus MALDI-TOF MS) a number of the raft proteins identified in this study, including some membrane-associated mitochondrial proteins, were also recently reported to co-purify with lipid rafts in T-cells [41].

Although some of the mitochondrial proteins listed in Table 2 have been described in association with either the plasma membrane (VDAC1 and VDAC2) [42] or surface receptors (prohibitin) [43], the other proteins of this group are confined to mitochondria, raising the intriguing possibility of the existence of mitochondrial 'rafts'. In this context, both glycosphingolipids and polysialogangliosides have been reported to be associated with mitochondria [44]. Furthermore, similarly to lipid raft-associated proteins, mitofilin is resistant to detergent extraction [38]. Of note, Bcl-2, which is associated with the outer mitochondrial membrane, failed to co-purify with lipid rafts (Figure 1). Furthermore, the immunoblot analysis presented in Figure 1 rules out an aspecific co-purification of proteins from cellular organelles, such as lysosomes, early endosomes or the Golgi apparatus, with the lipid raft preparations used in this study. Interestingly, the cluster analysis in Figure 4 shows that, with the exception of ATP synthase β, all integral mitochondrial membrane proteins, as well as NADH dehydrogenase, which interacts with the inner mitochondrial membrane [45], co-cluster

Table 2 Identification by MS of lipid raft-associated proteins

TIP47, tail-interacting protein of 47 kDa; HSC, heat-shock cognate protein; GRP, glucose-regulated protein; TOM, translocase of the outer mitochondrial membrane.

Spot no.	Accession no.	Name	Localization	Function	Method of identification	Reference
–	P06239	Lck	Plasma membrane*†	Signalling	Western blotting	[5]
–	P06241	Fyn	Plasma membrane*†	Signalling	Western blotting	[5]
1	–	GPI-1	Plasma membrane*†§	–	GPI labelling	
2	–	GPI-2	Plasma membrane*†§	–	GPI labelling	
3	–	GPI-3	Plasma membrane*†§	–	GPI labelling	
4	–	GPI-4	Plasma membrane*†§	–	GPI labelling	
5, 6	P02570	Actin β	Cytosol and membranes	Component of the cytoskeleton	Database, MS	
7	P04901	Gβ	Plasma membrane*†	Signalling by heterotrimeric G-proteins	MS	[28,48]
8	Q9UJZ1	Stomatin-like2	Plasma membrane and endosomes*†‡	Regulation of ion transport	MS	[29,49]
9	O60664	TIP47	Endosomes, interacts with mannose 6-phosphate receptor/Rab9	Mannose 6-phosphate receptor transport from endosomes to *trans*-Golgi network	MS	[30]
10, 11	P11142	HSC70	Intracellular and mitochondrial membranes	Molecular chaperone	MS	[31]
12	P38646	GRP75	Intracellular and mitochondrial membranes	Molecular chaperone	MS	[32]
13, 14	P35232	Prohibitin	Inner mitochondrial membrane interacts with sIgM, c-Raf and Rb	Molecular chaperone represses E2F transcription factors	MS	[37,43]
15, 16	P21796	VDAC1	Outer mitochondrial and plasma membranes‡	Control of apoptosis	MS	[42,50]
17–19	P45880	VDAC2	Outer mitochondrial and plasma membranes‡	Control of apoptosis	MS	[42,50]
20, 21	O96008	TOM40	Outer mitochondrial membrane‡	Import of protein precursors into mitochondria	MS	[34,35]
22	P06576	ATP synthase β	Inner mitochondrial membrane‡	Synthesis of ATP	MS	[36]
23	O75489	NADH dehydrogenase	Inner mitochondrial membrane, matrix face	Electron transport	MS	[40]
24–29	Q16891	Mitofilin	Inner mitochondrial membrane‡	Motor protein	MS	[38,39]
30	P47985	Ubiquinol-cytochrome *c* reductase complex iron-sulphur subunit	Inner mitochondrial membrane‡	Generation of electrochemical potential coupled to ATP synthesis	MS	[45]
31	P22695	Ubiquinol-cytochrome *c* reductase core protein	Inner mitochondrial membrane‡	Generation of electrochemical potential coupled to ATP synthesis	MS	[45]
32	Q9NX40	Ovarian carcinoma immunoreactive antigen	Not determined	Not determined	MS	
33	Q9NX63	Hypothetical protein FLJ20420	Not determined	Not determined	MS	

* Lipid raft-associated.
† Membrane association through post-translationally added lipid moiety.
‡ Integral membrane protein.
§ By analogy with known GPI-anchored proteins.

as a group of proteins with a similar profile of temporal variation, in support of a dramatic reorganization of mitochondrial membranes triggered by TCR occupancy.

Concluding remarks

Although lipid rafts have been implicated in a number of cellular functions, in lymphocytes lipid rafts have to date been studied mainly in the context of immunoreceptor signalling. It is now accepted that in T-cells lipid rafts are dynamic platforms where proteins implicated in the TCR signalling cascade are transiently recruited following receptor engagement. Recent data using the polyoxyethylene ether Brij-98 as detergent show that the TCR signalling-initiation machinery is actually preassembled in lipid rafts [46]. Protein association with lipid rafts is likely to be facilitated by the coalescence of rafts following TCR triggering, a process promoted by cortical actin reorganization and enhanced by engagement of co-stimulatory receptors such as CD28 [26,47]. Furthermore, CD28 co-engagement induces the transport to the cell surface of intracellularly stored lipid rafts [47].

Our functional proteomics approach underlines a potential link of lipid rafts with a number of additional functions involving cellular membranes in T-cells. Indeed, the data have revealed an unexpected dynamic pattern of intraction with lipid rafts not only of proteins participating in the TCR signalling cascade, but also of a wide array of proteins associated, directly or indirectly, with the plasma membrane and intracellular membranes and implicated in a variety of cellular functions. While the biological significance of the association with lipid rafts of the proteins identified requires clarification, this study provides a view into the deep and far-reaching changes induced in a cell by surface-receptor triggering. In addition, the data support the concept that cluster analysis might be used as a valuable descriptive and predictive tool in proteomics to identify classes of polypeptides potentially implicated in specific cellular functions.

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