

Polyunsaturated Eicosapentaenoic Acid Displaces Proteins from Membrane Rafts by Altering Raft Lipid Composition*

Received for publication, July 3, 2001, and in revised form, July 17, 2001
Published, JBC Papers in Press, August 6, 2001, DOI 10.1074/jbc.M106193200

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Polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (20:5 (*n*-3)) inhibit T lymphocyte activation probably by displacing acylated signaling proteins from membrane lipid rafts. Under physiological conditions, saturated fatty acyl residues of such proteins partition into the cytoplasmic membrane lipid leaflet with high affinity for rafts that are enriched in saturated fatty acyl-containing lipids. However, the biochemical alteration causing displacement of acylated proteins from rafts in PUFA-treated T cells is still under debate but could principally be attributed to altered protein acylation or changes in raft lipid composition. We show that treatment of Jurkat T cells with polyunsaturated eicosapentaenoic acid (20:5 (*n*-3)) results in marked enrichment of PUFAs (20:5; 22:5) in lipids from isolated rafts. Moreover, PUFAs were significantly incorporated into phosphatidylethanolamine that predominantly resides in the cytoplasmic membrane lipid leaflet. Notably, palmitate-labeled Src family kinase Lck and the linker for activation of T cells (LAT) were both displaced from lipid rafts indicating that acylation by PUFAs is not required for protein displacement from rafts in PUFA-treated T cells. In conclusion, these data provide strong evidence that displacement of acylated proteins from rafts in PUFA-treated T cells is predominantly due to altered raft lipid composition.

(3–7). Previous data (8) strongly emphasized that selective modification of functional membrane lipid microdomains domains, so-called rafts, underlie PUFA-mediated inhibition of T cell signal transduction.

Membrane lipid rafts are specialized regions within the plane of the plasma membrane and play an essential role in T cell signal transduction (9–12). Lipid microdomains are characterized by a high concentration of cholesterol and sphingolipids, such as sphingomyelin and glycolipids, and their polar lipids contain predominantly saturated fatty acyl residues (13–15). Such lipids spontaneously aggregate to form liquid-ordered membrane regions that are insoluble in non-ionic detergents facilitating raft isolation as detergent-resistant membrane domains (15, 16). Sphingolipids and glycosylphosphatidylinositol (GPI)-anchored proteins are typical constituents of rafts, but both are attached exclusively in the outer (exoplasmic) leaflet of the plasma membrane bilayer (16, 17). The lipid composition of the inner (cytoplasmic) leaflet of rafts is less well defined. It appears to be also enriched in saturated fatty acyl chains (18) but lacks stabilizing sphingolipids.

A variety of proteins are targeted into membrane rafts, mostly by means of covalent lipid modifications. Many proteins are associated with the extracellular side of plasma membrane rafts by means of a GPI anchor that partitions in the exoplasmic leaflet of the lipid bilayer. In contrast, transmembrane and intracellular proteins are targeted to membrane domains by acylation with fatty acyl moieties (19). Myristoylation of the N-terminal amino group by amide bondage occurs co-translationally and is irreversible. However, for attachment to the plasma membrane (20, 21) and localization in membrane lipid domains, proteins require at least a second fatty acyl substitution on cysteines usually with palmitoyl residues (22, 23). Protein S-acylation is reversible and may be used to regulate the subcellular localization of proteins (19, 22–25). The high packing order of saturated myristoyl and palmitoyl moieties facilitates interactions with membrane subdomains in a liquid-ordered state (26), and this preferential distribution underlies the enrichment of acylated proteins in lipid rafts under physiological conditions (19, 25).

Numerous proteins involved in T cell signaling such as intracellular Src family protein-tyrosine kinases and transmembrane linker for activation of T cells (LAT) are highly concentrated in rafts due to post-translational palmitoylation (27–30). The localization of these proteins in rafts is important for their function emphasizing the role of lipid rafts in T cell signaling (11, 12). Treatment of T cells with PUFAs selectively displaces signaling proteins such as Src family kinases Lck and Fyn from lipid rafts, which under physiological conditions are attached to the cytoplasmic membrane lipid leaflet by means of acyl moieties (8). On the other hand, GPI-anchored proteins and glyco-

Polyunsaturated fatty acids (PUFAs),¹ particularly eicosapentaenoic acid (20:5 (*n*-3)) and other members of the *n*-3 series that are abundant in marine fish oils, modulate the immune response and are therefore applied clinically as adjuvant immunosuppressant in the treatment of inflammatory disorders (1, 2). The immunomodulatory action of PUFAs is associated with their ability to suppress T cell activation and function

* This work was supported by Austrian Science Foundation Grant P13507-Med (to T. M. S.), the ICP Program of the Austrian Federal Ministry for Education, Science, and Culture (to T. M. S. and W. W.), and Academy of Sciences of the Czech Republic Grant A7052904 (to P. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¹ The abbreviations used are: PUFA, polyunsaturated fatty acid; EI-MS, electron-impact ionization mass spectrometry; ESI-MS, electrospray ionization mass spectrometry; GPI, glycosylphosphatidylinositol; HD, high density fractions; ID intermediate density fractions; LAT, linker for activation of T cells; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PAGE, polyacrylamide gel electrophoresis.

sphingolipids that are both attached to the exoplasmic membrane leaflet remain unaffected. This modification of rafts by PUFAs is strongly related to their ability to block protein tyrosine phosphorylation and calcium response in T cells and was therefore proposed to underlie the inhibitory effect of PUFAs on cell signaling (8).

The biochemical alterations underlying the selective displacement of acylated proteins from rafts by PUFA treatment are still under debate but could principally be attributed to altered protein acylation or changes in the raft lipid environment. We show that treatment of Jurkat T cells with polyunsaturated eicosapentaenoic acid (20:5 (*n*-3)) considerably increases raft lipid unsaturation by enrichment of PUFAs in raft lipids including lipids preferentially residing in the cytoplasmic membrane lipid leaflet, thereby making the formation of liquid-ordered domains unlikely (26). Furthermore, acylated proteins were displaced from lipid rafts in PUFA-treated T cells despite acylation with palmitate or its metabolite. In conclusion, these data provide strong evidence that displacement of acylated proteins from rafts by PUFA treatment is mainly due to changes in lipid composition of membrane rafts rather than altered protein acylation.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were purchased from Sigma unless stated otherwise. The following antibodies were used: mouse monoclonal antibodies LCK-01 (anti-Lck) for immunoprecipitation; anti-Lck for immunoblotting from Transduction Laboratories (Lexington, KY); polyclonal rabbit anti-LAT from Upstate Biotechnology, Inc. (Lake Placid, NY); horseradish peroxidase-labeled goat anti-mouse IgG from Bio-Rad; horseradish peroxidase-labeled goat anti-rabbit IgG from Accurate Chemicals (Westbury, NY).

Cell Culture and Fatty Acid Treatment—The human T cell line Jurkat E6-1 (American Type Culture Collection, Manassas, VA) was grown under standard conditions in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (HyClone, Logan, UT), penicillin/streptomycin (50 units/ml and 50 µg/ml, respectively; Life Technologies, Inc.), and 2 mM glutamine (Life Technologies, Inc.). For modification of cellular lipids, cells were incubated for 2 days in serum-free Iscove's modified Dulbecco's medium (Life Technologies, Inc.), supplemented with 0.4% (w/v) bovine serum albumin (fraction V, containing less than 3 µM total fatty acids), 1 mg/liter transferrin, 8.1 mg/liter monothiolglycerol, and glutamine and antibiotics as above (31), with addition of 50 µM of either polyunsaturated eicosapentaenoic acid (20:5 (*n*-3)) or stearic acid (18:0), which served as a control, as described (8). Although stearic acid can provoke a moderate shift in bulk membrane fatty acid content from 16:0 to 18:1, stearic acid was considered the most suitable fatty acid to be added to control cultures since non-essential fatty acids are highly abundant in human serum, and this fatty acid was previously shown not to influence cellular PUFA content and membrane subdomain distribution of proteins compared with controls treated with vehicle only (ethanol ≤0.5%) (8).

Isolation of Rafts—Lipid rafts were isolated from fatty acid-treated Jurkat T cells as described (8). Briefly, cells were washed three times in Hanks' balanced salt solution (Life Technologies, Inc.) including 10 mM Hepes (pH 7.4; buffer A), swollen in hypotonic buffer (42 mM KCl, 5 mM MgCl₂, 10 mM Hepes, pH 7.4) in the presence of protease inhibitors (1 µM pepstatin, 10 µg/ml aprotinin (Bayer, Leverkusen, Germany), 5 mM iodoacetamide, 10 µg/ml leupeptin, and 0.4 mM pefabloc (Roche Molecular Biochemicals)), and mechanically broken. Membranes were pelleted from post-nuclear supernatants as described (8, 32). After resuspending these "bulk membranes" in buffer B (20 mM Tris-HCl, pH 8.2, 140 mM NaCl), 20% of the volume was set aside for later analyses, and membranes were lysed in 0.5 ml of lysis buffer consisting of buffer B including 1% Brij-58 (Pierce) and protease inhibitors (31). Lysates were adjusted to 40% (w/v) sucrose, placed under a 5–20% linear sucrose gradient in buffer B with protease inhibitors. Following ultracentrifugation (345,000 × *g*, 18 h, 4 °C), 400-µl fractions were collected from the top. Rafts were recovered from fractions 4–7, whereas proteins from soluble membrane fractions were found in intermediate (ID; combined fractions 8 and 9) and high density fractions (HD; combined fractions 10 and 11) as characterized in detail previously (8).

Fatty Acid and Cholesterol Analysis—Raft-containing density gradient fractions 4–7 were combined and diluted 5 times with buffer B, and

rafts were pelleted (100,000 × *g*, 30 min, 4 °C). Rafts and bulk membranes prepared in parallel from PUFA and control-treated T cells were extracted and transesterified to methyl esters by a one-step reaction as published (33) with minor modifications. Briefly, samples were freeze-dried overnight before addition of 2 ml of methanol (Merck)/benzene (Riedel-de Haën, Seelze, Germany), 4:1 (v/v), including 100 ng of heptadecanoic acid as internal standard. 200 µl of acetyl chloride (Fluka, Buchs, Switzerland) was added and methanolysis performed by incubation for 60 min at 100 °C under continuous stirring. The reaction was stopped by addition of 5 ml of 6% K₂CO₃, and the organic phase was collected. Fatty acid methyl esters were separated by chromatography on a DB-23 (J & W Scientific, Folsom, CA) as suggested by the manufacturer. Fatty acid methyl esters of highest available quality (Sigma) were used as standards.

The lipid extracts prepared for fatty acid analysis were also used for detection of cholesterol by gas chromatography and electron-impact ionization mass spectrometry (EI-MS, *m/z* 386, M⁺). Lipid extracts were directly injected in a Hewlett-Packard (Boise, ID) GC-MS 5973 system equipped with a CP Sil5 25 m-0.2 mm-0.12 µm capillary column (Chrompack, Middleburg, Netherlands). Gas chromatography was operated at 80 °C for 30 s, 80–185 °C at 30 °C/min, 185–250 °C at 1 °C/min with constant flow (1.1 ml/min) of helium gas. EI-MS was run at 70-eV filament voltage. Heptadecanoic acid (*m/z* 284, M⁺) was detected by EI-MS as internal standard in parallel with cholesterol. Cholesterol and heptadecanoic acid were identified by retention time and mass to charge values and quantified by their ion intensity compared with standards. Since heptadecanoic acid served as internal standard for both fatty acid and cholesterol quantitation, the amount of cholesterol was expressed as mol % of the sum of all fatty acids, which was used as an estimate of the total amount of the lipid phase of the membrane. The significance of differences in fatty acid and cholesterol content between rafts and bulk membranes or PUFA- and control-treated cells, respectively, was calculated by paired Student's *t* test, and according to Bonferroni's correction for multiple testing, a *p* < 0.025 was considered statistically significant.

Mass Spectrometry of Raft Phospholipids—Total lipids from rafts were extracted (34) and separated into neutral lipids, fatty acids, and polar lipids by thin layer chromatography on silica gel 60W plates with aluminum backing (Merck) using hexane/diethyl ether/acetic acid (70:30:1). Polar lipids were extracted with methanol/chloroform/water (50:25:20) and were finally dissolved in acetonitrile/water/formic acid (50:50:0.1) for introduction in the mass spectrometer by flow injection (35). Electrospray ionization-mass spectrometry (ESI-MS) was performed on a VG Quattro mass spectrometer (Micromass, Manchester, UK) connected to a Jasco PU-980 HPLC pump (0.1 ml/min), a Jasco LG-980-02 Ternary Unit (Jasco, Japan), and a VICI 10 port injection valve with 20-µl sample loop (VICI AG, Switzerland). Cone voltage was operated at 65 V and capillary voltage at 4.5 kV in positive mode. Calibration and sample injection was performed as described (35). Dimyristoylphosphatidylcholine was used as internal standard to quantify phospholipids according to their relative ion intensity (36–38). Phosphorus content of raft lipids was determined as described (39), and data were expressed as nanograms of dimyristoylphosphatidylcholine equivalents/ng of phosphorus. Most probable individual fatty acyl substitutions given in parentheses were assigned to phosphatidylcholine (PC) and phosphatidylethanolamine (PE) according to their relative abundance in total raft fatty acids. Differences in phospholipids species between PUFA-treated and control groups were tested by one-tail unpaired Student's *t* test, and a *p* value < 0.05 was considered statistically significant.

Analysis of Protein Palmitoylation—Fatty acid-treated cells were washed in buffer A, and 10⁸ cells were incubated for 4 h in Iscove's medium supplemented as above including 100 µCi/ml [9,10-³H]palmitic acid (40–60 Ci/mmol; Amersham Pharmacia Biotech) in ethanol (final concentration 0.25% (v/v)). Labeled cells were washed twice in medium including 0.5% fatty acid-free bovine serum albumin and twice in buffer A before preparation of membranes and isolation of lipid rafts as detailed above. Octyl glucoside was added to density gradient fractions (rafts, ID, HD) at 60 mM final concentration to completely solubilize raft proteins (40). Proteins were immunoprecipitated from density gradient fractions (rafts, ID, HD) either by LCK-01 antibody directly bound to Eupergit beads (Röhm Pharma Polymer, Darmstadt, Germany) or by GammaBind Plus beads (Amersham Pharmacia Biotech) preincubated with anti-LAT antibody, respectively. Immunoprecipitates were washed and resuspended in 25 µl of non-reducing sample buffer, and proteins were resolved by SDS-PAGE for detection of radioactive labeling and specific proteins, respectively. For fluorography, gels were fixed in acidic acid (10%)/methanol (30% (v/v)), incubated in enhancer (En³hance, DuPont), and exposed to preflashed films (X-Omat AR,

TABLE I
Raft and bulk membrane fatty acid composition from control (18:0) and PUFA(20:5 (n-3))-treated T cells

Fatty acids	18:0		20:5 (n-3)		20:5 vs. 18:0	
	Rafts	Membranes	Rafts	Membranes	Rafts	Membranes
14:0	0.9 ± 0.0 ^a	1.0 ± 0.1	1.9 ± 0.1	1.9 ± 0.2	***	***
16:0	24.6 ± 1.2 ^{*b}	18.6 ± 1.2	46.3 ± 2.5 ^{***c}	35.4 ± 2.2	*** ^d	*** ^d
16:1 (n-7)	0.8 ± 0.1	2.0 ± 0.2	0.7 ± 0.2	2.1 ± 0.2		
18:0	25.2 ± 0.9 ^{***}	19.6 ± 0.7	18.9 ± 1.0 ^{**}	12.4 ± 0.3	***	***
18:1 (n-9)	29.3 ± 1.1 ^{***}	45.4 ± 0.7	7.9 ± 0.3 ^{***}	13.2 ± 0.3	***	***
18:2 (n-6)	0.7 ± 0.0 ^{***}	1.3 ± 0.1	0.7 ± 0.0 ^{***}	1.8 ± 0.1		***
18:3 (n-6)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0		*
18:3 (n-3)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0		
20:0	0.4 ± 0.0 ^{***}	0.2 ± 0.0	0.3 ± 0.0 ^{**}	0.1 ± 0.0		*
20:1 (n-9)	1.1 ± 0.1	1.5 ± 0.2	0.3 ± 0.0 ^{***}	0.5 ± 0.0	***	***
20:2 (n-6)	2.1 ± 0.1	2.2 ± 0.1	0.2 ± 0.0 ^{***}	0.4 ± 0.0	***	***
20:3 (n-6)	1.5 ± 0.1 ^{**}	1.2 ± 0.1	0.8 ± 0.1 ^{***}	1.2 ± 0.1	***	***
20:4 (n-6)	4.9 ± 0.2 ^{**}	3.9 ± 0.2	0.8 ± 0.1 ^{***}	1.4 ± 0.1	***	***
20:5 (n-3)	0.1 ± 0.0	0.1 ± 0.0	4.5 ± 0.2 ^{***}	9.4 ± 0.6	***	***
22:0	0.9 ± 0.1 ^{***}	0.3 ± 0.1	0.2 ± 0.1	0.0 ± 0.0	***	**
22:1 (n-9)	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0 ^{**}	0.1 ± 0.0		
22:4 (n-6)	2.7 ± 0.3 ^{***}	1.2 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	***	**
22:5 (n-3)	1.0 ± 0.1 ^{***}	0.3 ± 0.0	14.0 ± 1.3 [*]	18.6 ± 2.2	***	***
22:6 (n-3)	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.2	0.1 ± 0.0		
24:0	1.3 ± 0.3 ^{**}	0.3 ± 0.1	0.6 ± 0.1 [*]	0.0 ± 0.0	**	**
24:1 (n-9)	1.9 ± 0.3 ^{***}	0.6 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	***	
Saturated fatty acids	53.5 ± 0.6 ^{***}	40.2 ± 0.5	68.3 ± 1.7 ^{***}	50.0 ± 2.4	**	*
Monounsaturated fatty acids	33.4 ± 0.8 ^{***}	49.7 ± 0.5	9.7 ± 0.3 ^{***}	16.3 ± 0.4	***	***
PUFAs	13.1 ± 0.6 ^{**}	10.2 ± 0.5	22.0 ± 1.7 ^{**}	33.7 ± 2.8	**	***
Unsaturation index	0.80 ± 0.02 ^e	0.83 ± 0.01	1.14 ± 0.08 ^{**}	1.74 ± 0.13	*	***
Mean chain length	18.04 ± 0.08 ^f	17.85 ± 0.07	17.81 ± 0.14 ^{**}	18.23 ± 0.15		*
Cholesterol	17.6 ± 0.6 ^{***g}	5.1 ± 0.2	18.1 ± 1.3 ^{***}	7.4 ± 1.0		

^a Jurkat T cells were treated with polyunsaturated eicosapentaenoic acid (20:5 (n-3)) or stearic acid (18:0), which served as a control, followed by preparation of membranes. Rafts were isolated, and fatty acid composition was analyzed in parallel with aliquots spared from bulk membranes. Fatty acid composition from five experiments is expressed in mol % (mean ± S.E.).

^b Asterisks in this column indicate significant differences compared with bulk membranes of 18:0-treated cells (*, $p < 0.025$; **, $p < 0.01$; ***, $p < 0.001$).

^c Asterisks in this column indicate significant differences compared to bulk membranes of 20:5 (n-3)-treated cells as detailed in Footnote b.

^d Asterisks in these columns indicate significant differences between rafts or bulk membranes, respectively, from 20:5 (n-3) versus 18:0-treated T cells as detailed in Footnote b.

^e Average number of double bonds per fatty acyl residue.

^f Average number of carbon atoms of fatty acyl residues.

^g Number of cholesterol molecules per 100 fatty acyl residues (mol %).

Eastman Kodak Co.) for 2–6 months at -80°C . Proteins were detected by standard immunoblotting procedures using chemiluminescence, and blots were exposed on a Lumi-Imager (Roche Molecular Biochemicals). For determination of overall palmitoylation, Lck was immunoprecipitated from whole cell lysates. Data for quantitative assessment were obtained by densitometric scanning of fluorography films and directly from Lumi-Imager files, respectively. Relative amounts of protein or palmitate-labeled proteins, respectively, recovered from soluble membrane fractions (ID, HD) were compared between PUFA- and control-treated T cells by unpaired two-tail Student's t test.

Subcellular Fractionation—Post-nuclear supernatants were prepared from fatty acid-treated cells as detailed above and subjected to centrifugation ($100,000 \times g$, 30 min, 4°C) in a tabletop ultracentrifuge Optima TL (Beckman Instruments, High Wycombe, Buckinghamshire, UK). After carefully separating the supernatant (S100), P100 pellets were resuspended in the original volume of buffer B, and samples were separated by SDS-PAGE for immunoblotting of Lck.

RESULTS

Treatment Alters Raft Lipid Fatty Acyl Composition—Treatment with polyunsaturated eicosapentaenoic acid grossly alters bulk membrane fatty acyl composition in T cells by incorporation of PUFAs and their elongated/desaturated derivatives (8). In order to investigate whether displacement of acylated proteins from membrane rafts could be due to altered raft lipid environment, we isolated rafts from PUFA (20:5 (n-3)) and control (18:0)-treated Jurkat T cells, and we assessed fatty acyl composition of rafts in parallel with bulk membranes (Table I). Rafts from control-treated cells were particularly enriched in saturated palmitic (16:0) and stearic acid (18:0), whereas monounsaturated palmitoleic (16:1 (n-9)) and oleic acid (18:1 (n-9))

were decreased compared with bulk membranes. When T cells were treated with polyunsaturated eicosapentaenoic acid (20:5 (n-3)), this PUFA and even more its elongation product docosapentaenoic acid (22:5 (n-3)) were highly enriched not only in bulk membranes but also in isolated rafts when compared with membranes or rafts, respectively, from control-treated cells. Notably, the concentration of saturated palmitic (16:0) and myristic acid (14:0) was also increased in PUFA-treated membranes and rafts. Rafts from PUFA-treated cells contained significantly less PUFAs (20:5 (n-3), 22:5 (n-3)) but more saturated fatty acids (16:0, 18:0) compared with bulk membranes. The unsaturation index was considerably increased by 42% in rafts from PUFA-treated compared with control-treated T cells (1.14 versus 0.80 double bonds per fatty acyl residue). However, the unsaturation index was significantly less in rafts compared with bulk membranes from PUFA-enriched T cells. Cholesterol was about three times enriched in rafts of T cells compared with bulk membranes, but there were no significant differences in cholesterol content of rafts or membranes following PUFA treatment. In summary, PUFAs are effectively incorporated in raft lipids and significantly alter the lipid environment of rafts even though to a lesser extent compared with bulk membranes.

PUFA Treatment Alters Acyl Substitution of Raft Lipids Residing in the Exoplasmic as Well as the Cytoplasmic Membrane Lipid Leaflet—PUFA-induced displacement of proteins from rafts selectively affects cytoplasmic and transmembrane proteins that are concentrated in rafts due to post-translational modification with fatty acyl moieties (8). Protein acyl moieties

TABLE II
PUFA-containing raft lipids from control(18:0) and PUFA(20:5 (n-3))-treated T cells

Raft lipids	18:0	20:5 (n-3)
Sphingomyelin		
20:5	21.2 ± 8.0 ^a	33.6 ± 12.0*
22:5	2.0 ± 0.7	3.5 ± 1.1*
22:6	1.8 ± 1.3	2.4 ± 1.1
Phosphatidylcholine		
36:5 (16:0/20:5)	1.8 ± 1.0	2.1 ± 1.0
38:5 (16:0/22:5, 18:0/20:5)	8.8 ± 4.3	11.0 ± 2.4
38:6 (18:1/20:5)	1.7 ± 0.5	4.6 ± 1.1***
40:5 (18:0/22:5)	21.5 ± 9.0	32.1 ± 11.8 ^b
40:6 (18:1/22:5)	3.2 ± 1.1	4.6 ± 1.5*
Phosphatidylethanolamine		
36:5 (16:0/20:5)	22.9 ± 6.3	30.0 ± 6.6*
38:5 (16:0/22:5, 18:0/20:5)	5.5 ± 3.3	7.5 ± 1.9
38:6 (18:1/20:5)	7.3 ± 4.4	9.8 ± 2.2
40:5 (18:0/22:5)	10.4 ± 3.7	16.3 ± 5.4*
40:6 (18:1/22:5)	23.2 ± 12.2	33.4 ± 9.2 ^c

^a Jurkat T cells were treated with polyunsaturated eicosapentaenoic acid (20:5 (n-3)) or stearic acid (18:0), which served as a control, followed by isolation of membrane rafts. Raft lipids were extracted and analyzed by ESI-MS. Raft lipids including PUFAs containing more than 5 double bonds are listed from 6 determinations and are expressed in nanograms of DMPC equivalents per ng of phosphorus (mean ± S.E.). Most probable individual fatty acyl substitutions are given in parentheses. Significant differences are indicated by asterisks.

^b $p = 0.06$.

^c $p = 0.07$.

partition in the cytoplasmic leaflet of the membrane lipid bilayer. If altered lipid environment underlies the displacement of acylated proteins from rafts, changes in membrane fatty acyl composition should affect membrane lipids residing in the cytoplasmic leaflet, whereas alterations of lipids from the exoplasmic leaflet could only indirectly affect membrane subdomain distribution of acylated proteins by interdigitating fatty acyl moieties. Therefore, we separately assessed incorporation of PUFAs in raft lipids typically localized in the exoplasmic (sphingomyelin, PC) or cytoplasmic (PE (41)) membrane lipid leaflet, respectively (Table II). PUFA-including phospholipid species were identified in raft lipid extracts by mass to charge values in ESI-MS spectra. PUFA treatment significantly increased concentrations of sphingomyelin substituted with 20:5 and 22:5 acyl chains, whereas 22:6, which cannot be synthesized from eicosapentaenoic acid by these cells (Table I (8, 42)), remained unchanged. In PUFA-treated T cells, PC species substituted with 38:6 (16:1/22:5) and 40:6 (18:1/22:5) acyl chains were markedly more abundant compared with control-treated cells, and 40:5 (18:0/22:5) showed a borderline significant elevation. Notably, substitution of PE with 36:5 (16:0/20:5) and 40:5 (18:0/22:5) acyl chains was significantly more abundant in rafts of PUFA-treated T cells in parallel with a borderline increase in 40:6 (18:0/22:5). Thus, PUFA treatment of T cells results in substantial enrichment of PUFAs in lipids residing in the exoplasmic as well as in the cytoplasmic leaflet of rafts. In conclusion, PUFA treatment elicits a considerably more unsaturated lipid environment in lipid rafts in general and particularly in their cytoplasmic lipid leaflet which could underlie raft displacement of acylated proteins in PUFA-treated T cells.

Protein Displacement from Lipid Rafts by PUFA Treatment Does Not Require Protein Acylation by PUFAs—Cytoplasmic proteins such as Src family kinase Lck, which are anchored to the plasma membrane by fatty acylation, require this modification for membrane attachment (29). When analyzing subcellular distribution of Lck, virtually all Lck was membrane-bound in PUFA (20:5) as well as control-treated T cells (18:0; Fig. 1A) indicating that *S*-acylation of Lck is not abolished by PUFA treatment. Accordingly, the cellular activity to palmitoy-

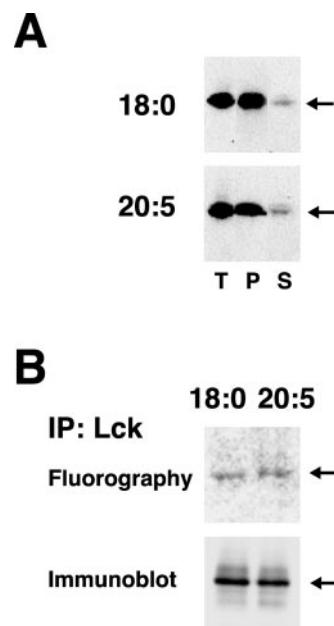


FIG. 1. Overall palmitoylation of [³H]palmitate-labeled proteins in fatty acid-treated T cells. A, Jurkat T cells were treated with 50 μM polyunsaturated eicosapentaenoic acid (20:5) or stearic acid (18:0), respectively. Post-nuclear supernatants (T, total) were separated into a P100 membrane fraction (P) and an S100 cytosolic fraction (S). Equivalent amounts were subjected to immunoblotting for detection of Lck protein. B, Jurkat T cells were treated with fatty acids as in A and labeled with [³H]palmitate as detailed under "Experimental Procedures." Src family kinase Lck was immunoprecipitated (IP) from whole cell lysates, and aliquots were separated by SDS-PAGE for detection of [³H]palmitate-labeled Lck by fluorography or Lck protein by immunoblotting, respectively. The position of Lck (p56) is marked (arrow).

late Lck was unaltered following PUFA treatment as indicated by incorporation of similar amounts of tritiated palmitate (Fig. 1B).

Cytoplasmic and transmembrane proteins that are enriched in lipid rafts are generally *S*-acylated with saturated fatty acids (particularly palmitate), but incorporation of unsaturated fatty acids may occur although with considerably lower efficiency. However, if protein *S*-acylation with PUFAs is a prerequisite for the displacement of proteins from rafts by PUFA treatment, proteins with attached palmitoyl residues must not be detectable outside rafts of PUFA-treated T cell membranes. Therefore, we analyzed the membrane subdomain distribution of proteins metabolically labeled with palmitate in eicosapentaenoic acid and control-treated T cells. In sucrose density gradients from control-treated T cells, Lck protein was found almost exclusively in raft fractions (18:0; Fig. 2, A and C). However, Lck protein was displaced from raft fractions to soluble membrane fractions of ID and HD in PUFA-treated cells as reported previously (Fig. 2, A and C, and Ref. 8). Notably, in parallel with its protein distribution in density gradients, a significantly larger proportion of palmitate-labeled Lck was recovered from soluble membrane fractions of PUFA-treated T cells (Fig. 2, A and C). In addition, similar to Lck, LAT (43), a transmembrane adaptor protein that is also palmitoylated under physiological conditions (30), was almost entirely located in rafts in control-treated T cells but was partially displaced from raft fractions by PUFA treatment in parallel with its palmitate-labeled form (Fig. 2, B and C). Thus, displacement of proteins from rafts occurs in PUFA-treated T cells even when proteins are acylated with palmitoyl residues or their derivatives. These data strongly suggest that altered raft lipid environment underlies protein displacement from rafts in PUFA-treated T cells rather than protein acylation with PUFAs.

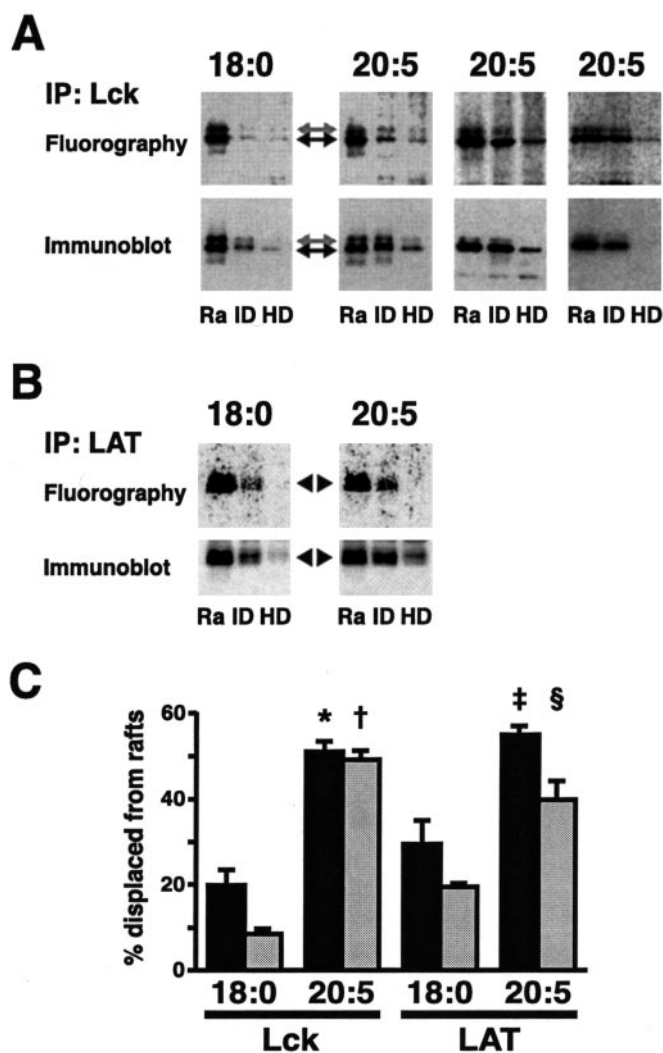


FIG. 2. Membrane subdomain distribution of [³H]palmitate-labeled proteins in fatty acid-treated T cells. *A*, fatty acid-treated Jurkat T cells were labeled with [³H]palmitate as in Fig. 1 and lysed in buffer containing 1% of the non-ionic detergent Brij-58. Rafts (*Ra*) and soluble membrane fractions of intermediate (*ID*) and high density (*HD*) were separated by sucrose density gradient centrifugation. Lck was immunoprecipitated (*IP*) from rafts and soluble membrane fractions, and aliquots were separated by SDS-PAGE for fluorography and immunoblotting, respectively. For PUFA-treated T cells (20:5), three independent examples are given showing membrane subdomain distribution of [³H]palmitate-labeled Lck and Lck protein. Positions of Lck migrating as p56 (black arrow) and p60 (gray arrow) are marked. *B*, fatty acid-treated Jurkat T cells were labeled with [³H]palmitate followed by membrane lysis and separation of rafts and soluble membrane fractions as in *A*. LAT was immunoprecipitated, and aliquots were separated by SDS-PAGE for fluorography and immunoblotting, respectively. The position of LAT migrating as p36/38 is marked by arrowhead. Representative results from 2 to 4 experiments are shown. *C*, quantitative assessment of experiments described in *A* and *B*. The proportion of Lck and LAT protein (black columns) and [³H]palmitate-labeled Lck and LAT (gray columns), respectively, which was displaced from rafts and detected in soluble membrane fractions (*ID*, *HD*) is given in percent (mean ± S.E.). Data from PUFA-treated cells (20:5) were compared with corresponding data from control-treated cells (18:0); *, $p < 0.001$ versus Lck protein in 18:0-treated T cells; †, $p = 0.001$ versus [³H]palmitate-labeled Lck in 18:0-treated T cells; ‡, $p < 0.05$ versus LAT protein in 18:0-treated T cells; §, $p < 0.05$ versus [³H]palmitate-labeled LAT in 18:0-treated T cells.

DISCUSSION

PUFAs inhibit T cell signaling most probably by displacing proteins that are palmitoylated under physiological conditions from lipid rafts. This displacement may principally be due to altered protein acylation and/or altered raft lipid environment.

By analyzing raft lipid fatty acyl composition and membrane subdomain distribution of palmitoylated proteins, we provide ample evidence that altered raft lipid composition underlies the displacement of proteins from lipid rafts in PUFA-treated T cells.

PUFAs were highly enriched not only in bulk membranes but also in rafts following PUFA treatment of T cells. Inclusion of unsaturated fatty acids in model membranes greatly reduces raft formation (26) resulting in detergent solubility of incorporated proteins (15, 44). Interestingly, raft lipids also contain some PUFAs under physiological conditions (18), and distinct PUFAs, *e.g.* arachidonic acid, were even enriched in rafts compared with bulk membranes (Table I). Changes in lipid composition induced by treatment with polyunsaturated eicosapentaenoic acid were markedly mitigated in rafts compared with bulk membranes, indicating that cells aim at maintaining raft structures by providing a saturated fatty acyl environment through yet unresolved compensatory mechanisms. Such partial compensation was particularly evident in the significantly minor incorporation of PUFAs and the concomitant increased content of saturated palmitic (16:0) and stearic acid (18:0) resulting in a markedly lower unsaturation index in rafts compared with the bulk membranes of PUFA-treated T cells (Table I). Nevertheless, incorporation of PUFAs resulted in a considerable 42% increase in unsaturation in raft acyl chains of membranes from PUFA-treated compared with control-treated T cells.

Acyl moieties of proteins partition in the cytoplasmic leaflet of the membrane lipid bilayer. Lipid alterations in the cytoplasmic lipid leaflet of membrane rafts would hence be particularly effective in displacing palmitoylated proteins. In addition, changes in fatty acyl composition of exoplasmic leaflet lipids (sphingomyelin and phosphatidylcholine) could contribute to the displacement of palmitoylated proteins from membrane rafts by interdigitation of acyl chains with cytoplasmic leaflet lipids or protein acyl moieties (16). Notably, the cytoplasmic leaflet of rafts incorporated significant amounts of PUFAs as shown by altered fatty acyl composition of raft phosphatidylethanolamine which predominantly resides in the cytoplasmic leaflet of the membrane bilayer (41) (Table II). Interaction of phospholipid acyl chains with cholesterol is required to maintain organization of membrane rafts in a liquid-ordered phase particularly in the cytoplasmic lipid leaflet that lacks sphingolipids (16). Raft cholesterol content was unaltered by PUFA treatment of T cells (Table I). However, the increase in lipid order induced by cholesterol highly depends on acyl chain unsaturation of phospholipid species in the absence of sphingolipids (45). The cholesterol-induced lipid order is particularly decreased in phosphatidylethanolamine and phosphatidylserine when substituted at its *sn*-2 position with PUFA compared with oleic acid (18:1) (45). Thus, changes in acyl chain composition of these typical lipids of the cytoplasmic leaflet as they occur in PUFA-treated T cells (Table II) appear detrimental for the lipid order in the cytoplasmic leaflet of membrane rafts, which is not stabilized by sphingolipids. In contrast, the exoplasmic lipid leaflet of rafts seems much less prone to be affected by fatty acyl unsaturation due to the presence of sphingolipids which greatly promotes the formation of a liquid-ordered state by interaction with cholesterol (26, 44, 46). In conclusion, the considerable changes in fatty acyl composition of raft lipids, particularly of those residing in the cytoplasmic lipid leaflet, provide a biochemical basis that could underlie the selective displacement of palmitoylated proteins from membrane rafts in PUFA-treated T cells.

Src family kinases Lck and Fyn as well as the transmembrane adaptor protein LAT are concentrated in rafts due to

S-acylation with saturated fatty acids, particularly palmitate. Accordingly, Src family kinases and LAT are displaced from lipid rafts by mutation of palmitoylation sites (27–30) or inhibition of protein palmitoylation by drugs (47). Protein S-acylation prefers but is not restricted to palmitoyl moieties (22, 23). Therefore, other saturated and even unsaturated fatty acyl moieties may be linked to cysteine residues of proteins (24, 48, 49). Proteins acylated with PUFAs have been shown *in vitro* not to target proteins to rafts despite efficient membrane anchoring (25). Thus, PUFA treatment of T cells could lead to substitution of protein palmitoyl residues by polyunsaturated fatty acyl moieties as suggested (50). To discriminate whether proteins are displaced from lipid rafts of PUFA-treated T cells by altered lipid environment or changes in protein acylation, we analyzed membrane subdomain distribution of palmitoylated Lck and LAT. Mammalian cells cannot synthesize PUFAs from palmitic acid. Therefore, if proteins are only displaced from lipid rafts when acylated with PUFAs then labeled proteins should not be found in soluble membrane fractions. In contrast, if protein displacement from rafts is due to altered raft lipid environment, palmitate-labeled proteins would be found in raft as well as soluble membrane fractions. The fact that palmitate-labeled Lck and LAT were recovered from soluble membrane fractions of PUFA-treated T cells to a similar extent as Lck and LAT protein (Fig. 2) reveals that direct protein acylation with PUFAs is not necessary for PUFA-mediated protein displacement from rafts. Moreover, these data strongly argue that alterations in the raft lipid environment are primarily responsible for displacement of acylated proteins from rafts following PUFA treatment. The reason for the minor importance of protein acylation with PUFAs may lie in its rather low efficiency compared with protein acylation with palmitate (50–52), whose concentration was even increased in bulk membranes of eicosapentaenoic acid-treated compared with control-treated T cells (Table I). Thus, although protein acylation by PUFAs is possible, it is unlikely that acylation with PUFAs is a major cause for protein displacement from lipid rafts in PUFAs-treated T cells.

In conclusion, our data on raft lipid fatty acyl composition and membrane subdomain distribution of palmitoylated proteins provide compelling evidence that incorporation of PUFAs in raft lipids is the leading alteration causing displacement of acylated proteins from membrane rafts in PUFA-treated T cells. Since raft localization of a variety of proteins is essential for their function in T cell signal transduction, alterations of raft lipid fatty acyl composition appear to mediate the inhibitory effect of PUFAs on T cell activation and possibly even their immunomodulatory effect.

Acknowledgments—We are grateful to Thomas Sigmund and Wolfgang Rafflesberg for skillful technical assistance and Hannes Stockinger for extensive discussion.

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