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GPI-microdomains (membrane rafts) and signaling of the multi-chain interleukin-2 receptor in human lymphoma/leukemia T cell lines

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Subunits (α, β and γ) of the interleukin-2 receptor complex (IL-2R) are involved in both proliferative and activation-induced cell death (AICD) signaling of T cells. In addition, the signaling β and γ chains are shared by other cytokines (e.g. IL-7, IL-9, IL-15). However, the molecular mechanisms responsible for recruiting/sorting the α chains to the signaling chains at the cell surface are not clear. Here we show, in four cell lines of human adult T cell lymphoma/leukemia origin, that the three IL-2R subunits are compartmented together with HLA glycoproteins and CD48 molecules in the plasma membrane, by means of fluorescence resonance energy transfer (FRET), confocal microscopy and immunobiochemical techniques. In addition to the β and γ_c chains constitutively expressed in detergent-resistant membrane fractions (DRMs) of T cells, IL-2Rα (CD25) was also found in DRMs, independently of its ligand-occupation. Association of CD25 with rafts was also confirmed by its colocalization with GM-1 ganglioside. Depletion of membrane cholesterol using methyl-β-cyclodextrin substantially reduced co-clustering of CD25 with CD48 and HLA-DR, as well as the IL-2 stimulated tyrosine-phosphorylation of STATs (signal transducer and activator of transcription). These data indicate a GPI-microdomain (raft)-assisted recruitment of CD25 to the vicinity of the signaling β and γ_c chains. Rafts may promote rapid formation of a high affinity IL-2R complex, even at low levels of IL-2 stimulus, and may also form a platform for the regulation of IL-2 induced signals by GPI-proteins (e.g. CD48). Based on these data, the integrity of these GPI-microdomains seems critical in signal transduction through the IL-2R complex.

Keywords: cytokine receptors; lipid rafts; cell proliferation; T lymphocytes; fluorescence energy transfer.

The multisubunit receptor of interleukin-2 cytokine (IL-2R) is essential in mediating T cell growth/clonal expansion [1] following antigen (or mitogen) stimulation, as well as in the control of activation-induced cell death (AICD) [2]. For IL-2 signaling, hetero-dimerization of the intracellular domains of β and γ_c chains was found critical [3], followed by Jak-assisted tyrosine-phosphorylation of downstream signaling molecules [eg. signal transducers and activators of transcription (STATs)] [4]. Interestingly, the 'common' γ subunit of IL-2R is shared by a number of other cytokine receptors (e.g. those of IL-4, IL-7, IL-9, IL-15) mediating diverse cellular responses [5,6]. This raises the question: how are the diverse α chains recruited/sorted to the signaling IL-2R β and γ_c chains? This question is further accentuated by the facts that the diverse α chains, in contrast to the signaling IL-2R β and γ_c chains, do not belong to the hemopoietin receptor superfamily, and their intracellular trafficking is different from that of the β and γ_c chains [7]. It is still not clear whether the assembly of the high affinity IL-2 receptor complex requires ligand occupation of CD25, as do other growth-factor receptors (such as EGF-receptor) [8]. The importance of these questions is also underlined by the recent success of immuno-toxin based cancer therapy targeting the α and β chains of IL-2R [9].

Recent FRET data, in contrast to an earlier 'sequential subunit-organization' (affinity conversion) model [10], suggested a preassembly of the three IL-2R subunits, even in the absence of their relevant cytokine ligands in the plasma membrane of T lymphoma cells. Binding of the physiological ligands (IL-2, IL-7, IL-15) was reported to selectively modulate the mutual molecular proximities/interactions of the IL-2R α, β and γ_c chains [11]. Microscopic (confocal fluorescence and immunogold labeling-based electron microscopy) studies revealed large scale (≈ 4–800 nm) overlapping clusters of CD25 and HLA molecules on T cell lines [12]. These observations all suggest that the above membrane proteins are somewhat compartmentalized in T cell plasma membranes.

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Abbreviations: IL, interleukin; AICD, activation-induced cell death; DRMs detergent-resistant membrane fractions; FRET, fluorescence resonance energy transfer; HTLV-I, human T cell lymphotropic virus I; HBSS, Hanks' balanced salt solution; STAT, signal transducer and activator of transcription.

Note: J. Matkó and A. Bodnár contributed equally to this work.

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Membrane compartmentation of T cell receptor with its co-receptors (CD4, CD8) and other signaling molecules (src kinases, LAT, etc.) by cholesterol- and glycosphingolipid-rich microdomains (rafts) has already been reported for T cells [13,14]. These lipid rafts were shown to preferentially accumulate GPI-anchored or double-acylated proteins (e.g. src kinase family), while the raft-targeting preference for transmembrane proteins still remains controversial and unclear [14,15], although a few examples of such proteins have been reported to associate with rafts (e.g. a fraction of LAT, CD4 and CD8 in T cells, CD44 in various cell types or influenza virus haemagglutinin in epithelial cells) [14].

Thus, the present study aimed at investigating whether the molecular constituents of the microscopically observed large (μm) scale clusters of CD25 [12] also display proximity (association) at the molecular (nm) scale. CD25 recruitment to the β and γ_c chains at the surface of human leukemia/lymphoma T cell lines was also studied with special attention to its ligand occupation. As lipid rafts (DRMs) can be considered as possible platforms of plasma membrane clustering of IL-2R chains, we investigated the relationship of IL-2R chains to T cell lipid rafts marked by CD48 GPI-anchored protein and the GM-1 ganglioside. Finally, we also investigated the relationship between membrane localization of the IL-2R complex and its signaling activity.

To probe cell surface protein organization, the distance-dependent fluorescence resonance energy transfer (FRET) method [16] was used [17–20], a technique that is very sensitive to molecular localization of membrane proteins on a submicroscopic distance scale of 2–10 nanometers. This is due to the inverse sixth power dependence of FRET efficiency on the actual distance between donor and acceptor dye-labels [19,21,22].

FRET data indicated a molecular level coclustering of the of IL-2R α, β and γ_c chains with the class I HLA, HLA-DR glycoproteins and the GPI-anchored CD48 molecule, similar on all the four distinct human T cell lines. Additional evidence (co-precipitation and co-capping with CD48, detergent-resistance analysis, colocalization with GM-1 lipid raft marker) has also shown supporting association of CD25 to lipid rafts, independent of its ligand occupation. Disintegration of rafts by cholesterol-depletion dispersed supramolecular clusters of CD25 with CD48 and HLA molecules. This compartmentalization may have functional implications, as disintegration of rafts also resulted in a remarkably reduced IL-2 stimulated tyrosine phosphorylation of T cell signaling molecules.

EXPERIMENTAL PROCEDURES

Cell lines and mAbs

The Kit225 K6 cell line is a human T cell with a helper/inducer phenotype and an absolute IL-2 requirement for its growth, while its subclone, Kit225 IG3, is IL-2 independent [23]. The IL-2 independent HUT102B2 cells were derived from a human adult T cell lymphoma associated with the human T cell lymphotropic virus I (HTLV-I) [24]. MT-1 is also an adult T cell leukemia cell line associated with HTLV-1 and is deficient in the signaling IL-2Rβ and γ subunits [25]. All cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum, penicillin and streptomycin [11]. To IL-2 dependent T cells, 20 $U \cdot mL^{-1}$ of recombinant interleukin-2 was added every 48 h. In some experiments, the cells were washed and then grown in IL-2-free medium for 72 h, and were therefore considered as T cells deprived of IL-2.

The subunits of the IL-2 receptor complex, class I HLA (A,B,C) and HLA-DR proteins were labeled with fluorescent dyes coupled to the following antibodies: IL-2Rα was targeted by anti-Tac Ig (IgG2a), while monoclonal anti-(Mik-β3) Ig (IgG1κ) and anti-TUGh4 Ig (Pharmingen, San Diego, CA, USA) were used against the IL-2Rβ and γ_c subunits, respectively. The following monoclonal antibodies were kindly provided by F. Brodsky (UCSF, CA, USA): W6/32 (IgG2aκ), specific for the heavy chain of class I HLA A,B,C molecules; L-368 (IgG1κ), specific for β2m; L243 (IgG_{2a}), specific for HLA-DR. The CD48 and the transferrin receptor (CD71) were tagged by MEM-102 (IgG1) and MEM-75 (IgG1), respectively (both from the laboratory of V. Horejsi). Fab fragments were prepared from IgG using a method described previously [19].

Aliquots of purified whole IgGs or Fab fragments were conjugated as described previously [26], with 6-(fluorescein-5-carboxamido) hexanoic acid succinimidyl ester (SFX) or Rhodamine Red™-X succinimidyl ester (RhRX) (Molecular Probes, Eugene, OR, USA). For labeling with sulfoindocyanine succinimidyl bifunctional ester (Cy3), a kit was used (Amersham Life Sciences Inc., Arlington Heights, IL, USA). Unreacted dye was removed by gel filtration through a Sephadex G-25 column. The fluorescent antibodies and Fabs retained their affinity according to competition with identical, unlabeled antibodies and Fabs.

Freshly harvested cells were washed twice in ice cold $NaCl/P_i$ (pH 7.4), the cell pellet was suspended in 100 μL of $NaCl/P_i$ (10^6 cells·mL^{-1}) and labeled by incubation with approximately 10 μg of SFX-, RhRX- or Cy3-conjugated Fabs (or mAbs) for 45 min on ice. The excess of mAbs was at least 30-fold above the K_d during the incubation. To avoid possible aggregation of the antibodies or Fab fragments, they were air-fuged (at 110 000 ***g***, for 30 min) before labeling. Special care was taken to keep the cells at ice cold temperature before FRET measurements in order to avoid unwanted induced aggregations of cell surface molecules or significant receptor internalization. Labeled cells were washed with cold $NaCl/P_i$ and then fixed with 1% formaldehyde. Data obtained with fixed cells did not differ significantly from those of unfixed, viable cells.

Measurement of fluorescence resonance energy transfer (FRET)

FRET measurements were carried out in a Becton–Dickinson FACStar Plus flow cytometer as described previously [17,26]. Briefly, cells were excited at 488 nm and 514 nm sequentially, and the respective emission data were collected at 540 and > 590 nm. Cell debris was excluded from the analysis by gating on the forward angle light scatter signal. Signals necessary for cell by cell FRET analysis and for spectral and detection sensitivity corrections were collected in list mode and analyzed as described previously [17,18]. Energy transfer efficiency (*E*) was expressed as a percentage of the donor (SFX) excitation energy tunneled to the acceptor (RhRX) molecules. The mean values of the calculated energy transfer distribution curves were used and tabulated as characteristic FRET efficiencies between the

two labeled protein epitopes. In the analysis of FRET, the uncertainties related to dye orientation [16] were overcome by using dyes with aliphatic C_6 spacer groups, allowing dynamic averaging of dipole orientations. Thus, the efficiency of FRET depended mostly on the actual donor–acceptor distance and the donor/acceptor ratio. When the two fluorescent labels are confined to two distinct membrane proteins, the dependence of FRET efficiency on the donor/acceptor ratio should also be taken into account [27,28]. In this case, measurements at different donor/acceptor ratios are necessary (as carried out in present experiments) and the normalized FRET efficiencies can be considered as estimates of the minimal fraction of acceptor–proximal donors.

Occasionally FRET was also detected on donor- and double-labeled cells by the microscopic photobleaching (pbFRET) technique [20], using a Zeiss Axiovert 135 fluorescent digital imaging microscope. Here, a minimum of 5000 pixels of digital cell images were analyzed in terms of bleaching kinetics and the efficiency of FRET was calculated from the mean bleaching time-constants of the donor dye measured on donor- and double-labeled cells, respectively [29].

Depletion of plasma membrane cholesterol by methyl-β-cyclodextrin (MβCD)

Freshly harvested T lymphoma cells (2×10^6 per mL) were treated with 7 mM MβCD for 45 min, at 37 °C, in Hanks' balanced salt solution (HBSS). (This treatment removes ≈40–50% of the plasma membrane cholesterol). The efficiency of cholesterol depletion was tested by measuring fluorescence anisotropy of 1,3,5,-diphenyl-hexatriene (DPH) lipid probe [30] in control and cyclodextrin-treated cells. For this test, cells were washed with HBSS and loaded with DPH (0.6 $\mu g \cdot mL^{-1}$) for 25 min, at 37 °C.

Isolation of detergent-resistant membrane fractions by sucrose gradient centrifugation

DRMs were isolated by equilibrium density-gradient centrifugation as described previously [31]. Briefly, Kit225 K6 T lymphoma cells were homogenized in ice cold TKM buffer (50 mM Tris/HCl, pH 7.4, 25 mM KCl, 5 mM $MgCl_2$, 1 mM EGTA) containing 73% (w/v) sucrose and 7 μL of protease inhibitor cocktail (1.5 $mg \cdot mL^{-1}$ aprotinin, 1.5 $mg \cdot mL^{-1}$ leupeptin, 1.5 $mg \cdot mL^{-1}$ pepstatin, 70 mM benzamidin, 14 mM diisopropyl fluorophosphate and 0.7% phenylmethanesulfonyl fluoride) in a 1-mL suspension of $\approx 10^8$ cells. This homogenate was incubated with 1% Triton X-100 or 15 mM Chaps on ice, for 20 min. Sucrose concentration was adjusted to 40% and the homogenate was placed at the bottom of an SW41 tube (Beckman Instruments, Nyon, Switzerland). It was overlaid with 6 mL of 36% and 3 mL of 5% sucrose in TKM buffer and centrifuged at 250 000 ***g*** for 18 h, at 4 °C, in a Centrikon T1180 ultracentrifuge (Kontron Instruments, Milan, Italy). The detergent-resistant, low-density membrane fraction was collected from the 5–36% sucrose interface where it formed a visible band.

Immunoprecipitation and Western-blot analysis

Aliquots of the cell lysate were mixed with antibody-precoated Protein G beads (50 μg mAb per 10 μL beads) and incubated overnight at 4 °C (10 μL beads was added to a cell lysate equivalent of 10^7 cells). After washing three times in detergent-free buffer, the samples were boiled in nonreducing SDS/PAGE sample buffer and the solubilized proteins were separated from the beads by centrifugation. Proteins precipitated with the applied antibody were analyzed by SDS/PAGE and Western blot techniques. Aliquots of DRMs were boiled in nonreducing SDS/PAGE sample buffer for 10 min. Proteins were separated electrophoretically on a Bio-Rad minigel apparatus (Bio-Rad, Richmond, VA, USA) and were transferred to nitrocellulose membranes (Pharmacia Biotech., San Francisco, CA, USA). Membranes blocked by Tween 20/NaCl/P_i containing low-fat dry milk powder were incubated with primary antibodies for 60 min in Tween 20/NaCl/P_i/1% BSA, washed three times in Tween 20/NaCl/P_i and incubated with horse radish peroxidase-conjugated secondary antibody [rabbit anti-(mouse IgG) Ig, Sigma, Steinheim, Germany] for an additional 1 h. After washing four times in Tween 20/NaCl/P_i and once in NaCl/P_i, the membranes were developed with ECL reagents (Pierce Chemicals, Rockford, IL, USA) and were exposed to an AGFA (Belgium) X-ray film.

Capping experiments

Control and MβCD-treated cells were labeled first either with Alexa488-conjugated anti-CD48 Ig (MEM102) or with RhRX-conjugated anti-CD25 Ig (Tac) on ice for 40 min, then incubated with anti-IgG (whole chain) RAMIG antibody at 37 °C, for 30 min. The cells were then fixed with formaldehyde, blocked with isotype control antibody and stained with the fluorescent antibody against the other protein, on ice. The double-stained cells were analyzed for cocapping by a Zeiss Axiovert 135 TV invert field fluorescence digital imaging microscope.

Detection of IL-2 stimulated tyrosine-phosphorylation of STATs

IL-2 induced tyrosine phosphorylation of STAT3 (and STAT5) was followed by flow cytometry as described previously for STAT1 [32]. Briefly, cells with or without IL-2 treatment were subjected to fixation and permeabilization (Fix&Perm Kit, Caltag Laboratories, Burlingame, CA, USA) and incubated (20 min) with specific rabbit anti-(STAT3/STAT5) Ig or rabbit polyclonal anti-(phospho-STAT3/STAT5) Ig (New England Biolabs, Inc., Beverly, MA, USA). These antibodies detect nonphosphorylated and phosphorylated Tyr moieties on STAT3/STAT5, respectively, without appreciable cross-reaction with other Tyr-phosphorylated STATs. After washing, cells were incubated with a second, FITC-conjugated anti-(rabbit IgG) Ig (DAKO/Frank Diagnostica, Hungary) for 30 min. After a final wash step, cells were resuspended in NaCl/P_i for flow cytometry.

RESULTS

IL-2R α, β, and γ_c chains exhibit nanometer scale supramolecular clusters with HLA glycoproteins and CD48 at the surface of T lymphoma/leukemia cells

For accurate proximity analysis by FRET, the expression levels of the three IL-2R subunits and the other mapped

proteins have been estimated on the four T cell lines by flow cytometry. The IL-2R α and γ_c chains were found constitutively expressed in several (6–10) thousands of copies in all cell lines, except in MT-1, which is deficient in α and γ chains. CD25 was expressed at a level eightfold to 14-fold higher than that of the α and γ chains on all the four T cell types ($\geq 10^5$ per cell), characteristic of leukemic or activated T cells. HLA-DR was abundant on all cell lines ($\geq 5 \times 10^5$ copies per cell). Surface density of class I HLA was low on MT-1 cells ($\approx 3 \times 10^4$ per cell), while very high ($\geq 10^6$ per cell) on the other three cell lines. Interestingly, class I HLA level detected by a conformation-specific mAb interacting with the α1/α2 domains of the heavy chain, W6/32, was approximately twice as high on T cells deprived of IL-2 than on cells growing in the presence of IL-2. This difference was not observed if L368 mAb against the β2-microglobulin light chain of class I HLA was used for detection (data not shown).

Then we analyzed plasma membrane topography of IL-2R subunits and HLA molecules by both flow cytometric [17–19] and microscopic photobleaching FRET (pbFRET) [20] techniques. Both FRET methods indicated a significant degree of molecular vicinity between CD25 and class I HLA molecules on all cells, regardless of the expression level of β and γ_c chains or class I HLA (see MT-1 cells; Fig. 1B). It is noteworthy that FRET between CD25 and the light chain (β2-microglobulin) of class I HLA was consistently weaker than the FRET between CD25 and the HLA heavy chain marked by anti-W6/32 Ig (data not shown). In addition to this, the signaling IL-2R β and γ_c chains in these cells also displayed molecular colocalization with class I HLA. Furthermore, all the three IL-2R chains showed similar locality to the HLA-DR molecules (Fig. 1B). The HLA glycoproteins (class I HLA and HLA-DR) also exhibited a high degree of homo- and hetero-association on all the four T cell lines (independent of class I HLA expresssion level), as assessed by FRET data (not shown). Significant FRET ($E \geq 12\%$) was measured also between CD25 and CD48 on these cell lines, while no FRET was detectable between CD48 and TrfR (CD71) (Table 1). Although microscopy failed to detect significant colocalization of CD25 with TrfR on large (μm) scale [12], FRET data ($E \approx 13\%$) suggest their partial colocalization on molecular (nanometer) scale, at the surface of these T cells.

The above molecular locality patterns could be observed in T cells of different growth phases and appeared similarly in Kit225K6 T cells growing in the presence of IL-2 or deprived of IL-2, alike. This strongly suggests that compartmentalization of the above proteins is an inherent (possibly microdomain-organization linked) property of the plasma membrane characteristic of these human leukemia/lymphoma T cell lines and it is not triggered by cytokine binding.

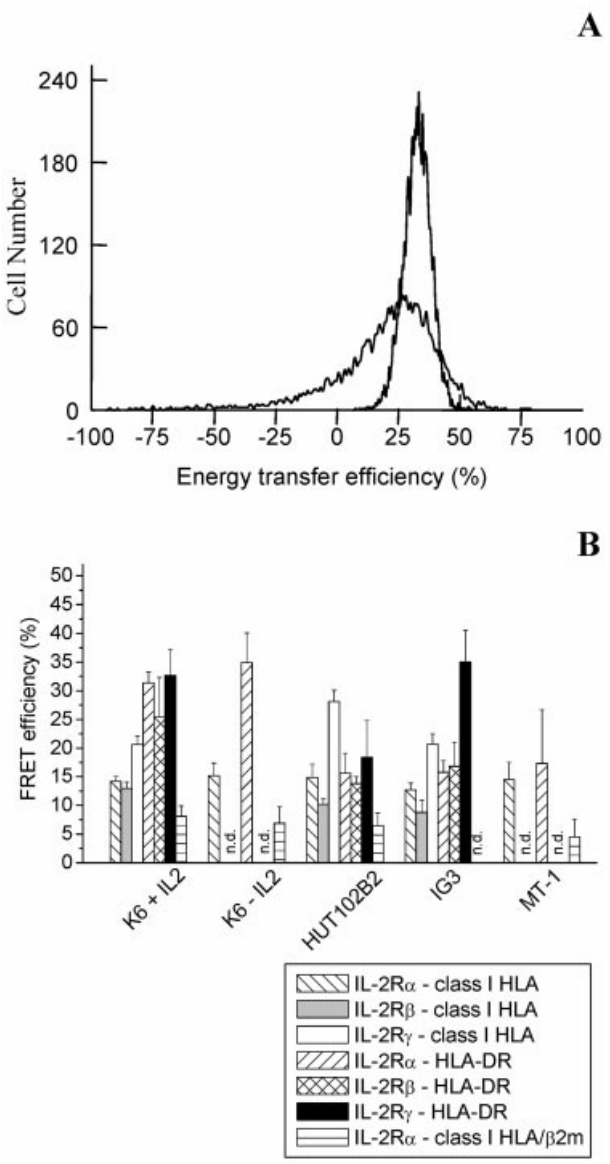

Fig. 1. FRET between IL-2R subunits and HLA glycoproteins in T leukemia and lymphoma cell lines. (A) Representative FRET efficiency (E, %) histograms measured on T lymphoma/leukemia cell lines, on cell-by-cell basis, using flow cytometry. The cell-independent intramolecular FRET between light and heavy chains of class I HLA (used as 'internal standard') (right, narrow distribution) and FRET between IL-2Rα and HLA-DR (left, broad distribution) are shown. (B) FRET efficiency data monitoring molecular associations of the IL-2R complex in four different human leukemia/lymphoma T cell lines. Bars represent mean FRET efficiencies ± SEM (n ≥ 3) between different pairs of protein epitopes (see legend), on the T cells indicated below the bars. n.d., not determined.

Association of IL-2R chains with GPI-microdomains (rafts) on T cell surfaces: evidence from detergent resistance, cocapping/coprecipitation with CD48 and colocalization with GM-1 ganglioside

Association of a protein with membrane rafts is usually defined biochemically by its presence in low density membrane fractions resistant to cold nonionic detergents [31,33]. Therefore, we investigated here whether the CD25 clusters mentioned previously are promoted by their association with DRMs, lipid rafts. Using immunoblotting, CD25 was detected in a significant amount in a low-density, detergent-resistant membrane fraction (DRM) of Kit225 K6 T cells after solubilization with nonionic detergents Triton X-100 (or Chaps, not shown) and the subsequent sucrose gradient centrifugation. The GPI-anchored CD48, as well as the signaling β and γ_c chains were also consistently detected in the same DRM (Fig. 2).

Table 1. FRET between raft and nonraft proteins: effect of cholesterol depletion by MβCD.

Cell Sample	Donor/ epitope	Acceptor/ epitope	FRET efficiency E (% ± SEM)
Kit225K6	CD48	CD25	12.6 ± 1.9
Kit225K6 + MβCD	CD48	CD25	2.3 ± 1.5
Kit225K6	CD25	HLA-DR	31.2 ± 0.9
Kit225K6 + MβCD	CD25	HLA-DR	16.3 ± 1.1
Kit225K6	CD25	CD71	13.6 ± 2.2
Kit225K6 + MβCD	CD25	CD71	14.1 ± 2.6
Kit225K6	CD48	CD71	2.1 ± 0.8
Kit225K6 + MβCD	CD48	CD71	1.9 ± 1.1

In order to see whether localization of CD25 in DRM depends on its ligand occupation, detergent-resistance analysis was simultaneously performed with the same T cells deprived of IL-2 (unoccupied IL-2R). CD25 and CD48 were similarly colocalized in DRMs of such cells, in a comparable amount, albeit a little less CD25 was found here in DRMs (Fig. 2). Thus, association of CD25 with detergent-resistant membrane fractions (DRMs) was defined by both Triton X-100 and Chaps detergents, and found approximately independent of the ligand (IL-2) occupation level of receptors on T cells.

Analysis of the whole sucrose gradient sedimentation profile led to some further conclusions. The transferrin receptor (CD71), believed to be a membrane protein excluded from lipid rafts [34,35], was not detectable in the 'light' DRM fractions of the cells, but localized in a higher density, soluble fraction of the sucrose gradient. This soluble fraction also contained CD25, in a comparable amount to that localized in DRMs. Much less CD48 was found in this fraction than in DRMs, according to the expectations (Fig. 2). This finding indicates that a substantial fraction of cell surface CD25 is associated with GPI microdomains, while the rest (approximately half of the cell surface CD25) is located in soluble membrane fractions, and thought to be distributed either randomly or associated with other membrane microdomains (e.g. those accumulating TrfR) at the surface of the T cell lines investigated.

Supporting the detergent-resistance data, CD25 and CD48 also exhibited a detectable, although weak, immuno-coprecipitation and cocapping in the plasma membrane of Kit225 K6 T cells (Fig. 3A,B). Additionally, confocal

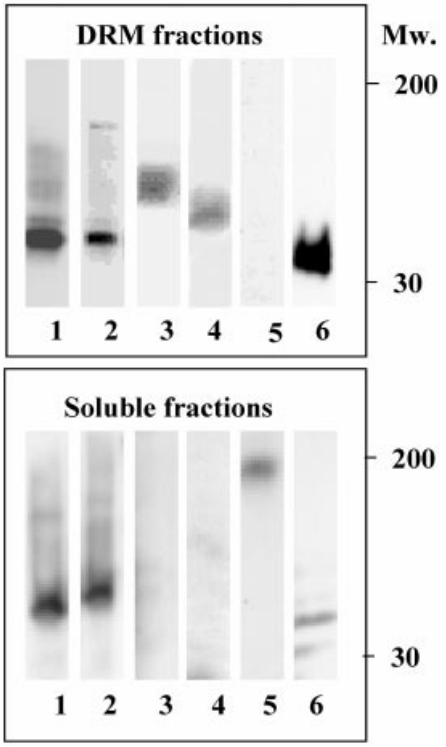

Fig. 2. Detergent resistance analysis of CD25, CD122 (IL-2Rβ), CD132 (IL-2Rγc), CD48 and CD71 (TrfR) in the plasma membrane of the human leukemia T cell line (Kit 225K6). Upper panel: Western blots of DRMs (obtained by Triton X-100 solubilization) from cells growing with or without (lane 2) IL-2 were developed by anti-CD25 Ig (anti-Tac Ig) (lane 1,2), MIKβ1 [anti-(IL-2Rβ) Ig] (lane3), TUGH4 [anti-(IL-2Rγ) Ig] (lane 4), anti-CD71 Ig (MEM-75) (lane 5) and anti-CD48 Ig (MEM-102) (lane 6). Lower panel: Western blot detection of CD25 in soluble membrane fractions of cells growing in the presence (lane 1) or absence (lane 2) of IL-2. The other four lanes were developed with antibodies corresponding to the samples shown in the appropriate upper lanes.

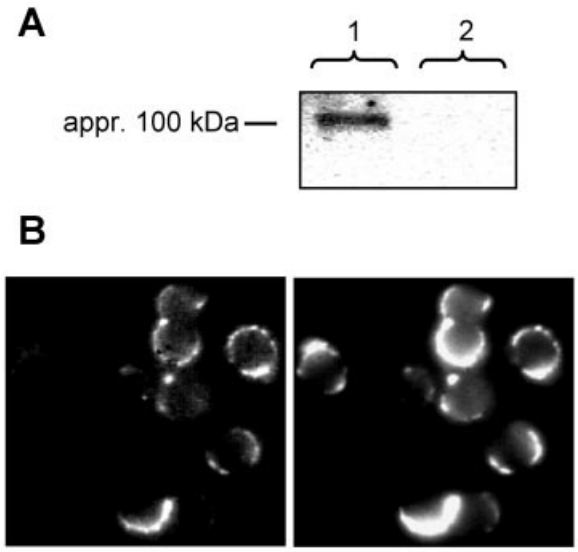

Fig. 3. Association of IL-2Rα (CD25) with lipid raft component CD48: evidence from coprecipitation and cocapping. Interaction of CD48 and CD25 in the plasma membrane of Kit225 K6 cells as revealed by immuno-coprecipitation. CD25 content of the cell lysate was immunoprecipitated by anti-Tac Ig. CD48 coprecipitated with CD25 was detected as described in Experimental procedures. Western-blot (nonreduced) was developed by MEM-102 (anti-CD48) Ig (lane 1) and an isotype-matched irrelevant mouse antibody (control) (lane 2). (B) Co-capping of CD25 and CD48 on Kit225 K6 cells. Details of the capping experiment is described in the Experimental procedures. Lane 1, black and white image of the green (Alexa488–anti-CD48 Ig) fluorescence of cells after capping. Lane 2, black and white image of the red (RhRX–anti-Tac Ig) fluorescence of the same cells. (Green fluorescence was detected using a 483 ± 15 nm excitation filter, a 500-nm dichroic mirror and a 518 ± 28 nm emission filter, while the red fluorescence was detected by a 548 ± 10 nm excitation filter, a 578-nm dichroic mirror and a 584-nm LP emission filter.)

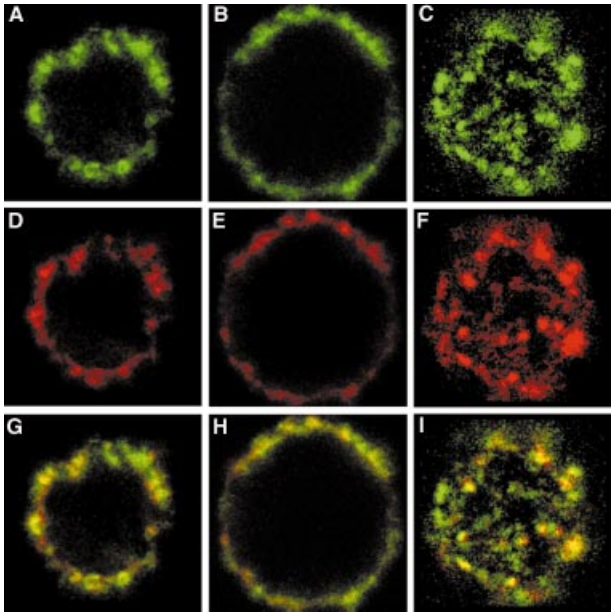

Fig. 4. Colocalization of CD25 with GM-1 ganglioside lipid raft marker labeled with FITC-cholera toxin B subunit on Kit225K6 T cells. Images of green and red fluorescence were collected in a Zeiss LSM 420 laser scanning confocal microscope (FITC-excitation: 488 nm; double dichroic: 488/543 nm; FITC-emission: 505–540 nm; Cy3-excitation: 543 nm; Cy3 emission: > 580 nm). Confocal images of double stained cells are shown at a 'close to bottom slice' (left column), at the 'middle cross section' (middle column) and at a 'top slice' (right column). The upper line (A, B, C) shows the fluorescence of FITC-CTX, the middle line (D, E, F) shows Cy3–anti-Tac Ig fluorescence and their pixel-registered overlays are shown in the bottom line of the figure (G, H, I). The yellow color in the overlay images represents membrane areas where the two labeled molecules are colocalized. (Field size: 15 × 15 microns; sampling: 512 × 512 pixels at eight bits.)

microscopic studies indicated a substantial level of colocalization of CD25 with GM-1, a lipid marker of rafts, labeled with fluorescent cholera toxin B subunit (CTX-B) (Fig. 4).

Clustering of IL-2R with CD48 and HLA glycoproteins in T cell membranes are cholesterol-sensitive

Disruption of the structural integrity of cholesterol/sphingolipid-rich microdomains is expected to abolish clustering of their protein constituents [21]. Therefore, two cell lines, the IL-2-dependent Kit225 K6 and the IL-2-independent HUT102B2 cells, were treated with water-soluble methyl-β-cyclodextrin (7 mM) to deplete their plasma membrane cholesterol [21,36]. Effect of cholesterol depletion on the microstructure of the plasma membrane was tested by measuring fluorescence anisotropy (r) of the DPH lipid probe sensing the orderedness/microviscosity of the membrane region in question. DPH fluorescence anisotropy remarkably decreased upon MβCD treatment in both cell lines (from 0.157 to 0.064 and from 0.149 to 0.082, respectively), reflecting a substantial membrane fluidization.

FRET on cholesterol-depleted T cell lines indicated largely decreased mutual vicinity between the IL-2Rα chains (CD25) and CD48 or HLA glycoproteins (Table 1.) Changes of similar tendency were observed on HUT102B2 cells, as well (data not shown). No FRET could be detected between CD48 and TrfR either before or after MβCD-treatment on either cell lines, suggesting that the membrane regions containing TrfR are physically separated from the microdomains accumulating clusters of CD25, CD48, HLA-DR and GM-1.

Disruption of raft integrity abrogates the IL-2 stimulated tyrosine-phosphorylation signals

Stimulation of T cells through the IL-2R complex results in heterodimerization of the intracellular domains of β and γ_c chains followed by association with Jak, Syk (or src family) kinases. These, in turn, phosphorylate the receptor chains, forming docking sites for further downstream signaling molecules, such as STAT transcription activation factors [2]. Cytokine-stimulation is usually followed by a number of tyrosine-phosphorylation events (e.g. phosphorylation of receptor chains or diverse downstream signal components, cross-phosphorylation of Jaks, etc.), while STAT3/STAT5 phoshorylation is thought to be a signal specific to IL-2 (and IL-15) stimulation [2,37]. As hetero-oligomerization and a proper orientation of IL-2R subunits seems essential to docking and activation of STATs, we investigated here whether raft integrity is a necessary condition to a proper transduction of cytokine-stimulated phosphorylation signals.

Figure 5A shows the time course of a developing overall tyrosine phosphorylation pattern stimulated by IL-2 in Kit225K6 T cells, as assessed by immunoblotting. The major tyrosine-phosphorylated bands appeared in the 35–60 kDa region and the extent of phosphorylation increased in time, plateauing in ≈ 15 min after IL-2 addition. The right panel of Fig. 5A clearly shows that pretreatment of the cells with cholesterol-extracting agent, MβCD, largely suppressed the extent of phosphorylation, nearly uniformly in the pattern.

Effect of cholesterol depletion on a signal step unique for IL-2 stimulation was also investigated. This was the tyrosine phosphorylation of STAT3/STAT5, monitored through binding of anti-(phospho-tyr-STAT3/STAT5) Ig. As Fig. 5B shows, stimulation of the Kit225K6 T cells with 1000 $U \cdot mL^{-1}$ IL-2 resulted in a largely enhanced binding of anti-(P-tyrSTAT3) Ig (more than fourfold) and anti-(P-tyrSTAT5) Ig (more than sixfold), respectively, relative to their basal level (detected in control, unstimulated T cells). This enhancement was remarkably abolished when the membrane cholesterol of T cells was depleted by MβCD before IL-2 stimulation (Fig. 5B).

DISCUSSION

To investigate the molecular background of large scale cell surface clusters/domains of HLA and IL-2R observed recently by fluorescence (confocal, SNOM) and electron microscopies [12,38,39], nanometer scale molecular localities of the α, β and γ_c chains of IL-2R, class I HLA and HLA-DR molecules were measured by FRET techniques. Earlier FRET studies on class I HLA–CD25 interaction have already been reported [24,40]. In addition to this, our data show that the signaling β and γ_c chains are also in close molecular proximity to both class I HLA and HLA-DR molecules in the plasma membrane of human T cell lines of

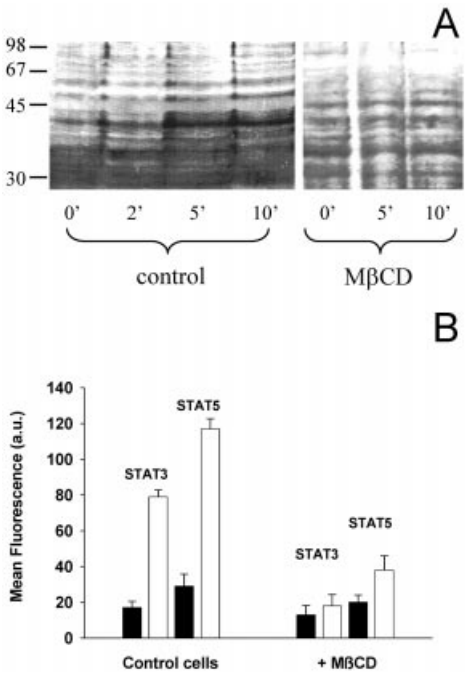

Fig. 5. Disruption of lipid rafts by cholesterol depletion abrogates IL-2 stimulated tyrosine-phosphorylation signals on T cells. (A) Detection of the overall tyrosine phosphorylation pattern in Kit225K6 T cells upon IL-2 stimulation. Parallel samples were obtained from cells pretreated with 7 mM MβCD. Aliquots were taken from the samples at the indicated times after IL-2 addition. After subjecting these aliquots to lysis, SDS PAGE and Western blotting, the membranes were incubated with horse-radish peroxidase conjugated antiphosphotyrosine antibody (ICN) and developed by ECL assay. (The X-ray films were digitized and normalized for the protein content of the membrane determined from amido-black absorbance. (B) Effect of cholesterol depletion on tyrosine phosphorylation/activation of STAT3/STAT5. The bars display means of flow cytometric fluorescence histograms of Kit225K6 T cells stained with FITC–anti-(rabbit IgG) Ig following binding of anti-(phosphotyrosine-STAT3) Ig or anti-(phosphotyrosine-STAT5) Ig. The data are displayed after subtraction of the background derived from isotype control staining and nonspecific binding of the second antibody. Error bars represent SEM values ($n \geq 3$). Black bars represent fluorescence proportional to binding of anti-(phospho-tyr-STAT3) Ig or anti-(phospho-tyr-STAT5) Ig in unstimulated cells, while white bars indicate its binding 15 min after IL-2 stimulation. Cell treatments are indicated below the abscissa.

leukemia/lymphoma origin. This might be characteristic of these cell lines overexpressing CD25 relative to resting peripheral T cells. FRET provided additional information about the possible interaction site between CD25 and class I HLA molecules in these protein clusters. The stronger FRET between CD25 and HLA-I heavy chain, compared with that between CD25 and β2 m indicates that CD25 preferentially interacts with the heavy chain of class I HLA. This is consistent with the altered binding of W6/32, but not of L368 Ig, after IL-2 deprivation of T cells. IL-2 binding to CD25 likely masks the W6/32 mAb binding site on proximal HLA molecules. Although the physiological significance of the molecular vicinity/association of class I HLA and IL-2R chains is still left undefined by these data, regulatory cross-talk suggested for the class I HLA–insulin receptor interaction [41] cannot be excluded.

Taken together, considering the simultaneous nature of FRET from IL-2R chains to HLA molecules and the 'cross-FRET' between IL-2R chains [11], the present data strongly suggest that at least a fraction of these molecules is compartmented in a common membrane microdomain. These supramolecular clusters may be characteristic of human leukemia/lymphoma T cell membranes, as immunogold staining of IL-2R on peripheral resting murine T lymphocytes and cell lines did not show any clustered distribution [42], in contrast to our recent microscopic results on leukemia/lymphoma cells [12].

On the other hand, a fraction of cell surface CD25 was found also proximal to transferrin receptors, thought to be located outside lipid rafts [34,35] in these T cells, as shown by previous [43] and present FRET data. As class I and class II HLA molecules were also found partially associated with TrfRs on T cells [43], our data may reflect that a fraction of cell surface CD25 molecules (low affinity form of IL-2R) is associated with TrfR-positive membrane microdomains, as well. Association of CD25 with these TrfR-positive domains may provide an efficient endocytosis/recycling pathway for the excess α chains (CD25) not involved in signal transduction of these cells.

Our data convincingly show that all the constituents of the high affinity human IL-2R are preferentially associated with DRMs (rafts) containing CD48, in T cells of leukemia/lymphoma origin. Constitutive expression of human IL-2R β and γ chains in membrane rafts was confirmed by our experiments, a result similar to that observed in mouse T lymphoma cells [44]. On the other hand, our data also support association of human CD25 with lipid rafts, independently of its ligand (IL-2) occupation. Our detergent-resistance data, in good agreement with earlier FRET data [11], suggest that the preassembly of the three IL-2R chains in the plasma membrane of T leukemia/lymphoma cells is not induced by ligand binding, as in case of other growth factor receptors (e.g. EGFR) [8].

Furthermore, our data suggest that the transient supramolecular assemblies of IL-2R chains, CD48, HLA-glycoproteins and GM-1 gangliosides at the cell surface are promoted by lipid 'raft' microdomains [33], which are rich in cholesterol and glycosphingolipids. These membrane microdomains were recently reported to be essential in compartmentation of signaling components providing efficient responses to TcR or IgE receptor activation [13–15,35]. In the T cells investigated here, raft-disruption by cholesterol-depletion resulted in a largely reduced molecular coclustering of IL-2R chains with CD48 and HLA-DR, possibly via lateral dispersion of these raft components. Although association of HLA molecules with lipid rafts, in general, is still a poorly understood and controversial issue [14,45], they may contribute to stabilize these microdomains by a 'fencing effect' [46], through their dynamic coupling to the cytoskeletal matrix [47,48], even if they are localized at the periphery of rafts.

Association of the IL-2R chains with lipid rafts (containing CD48) may have several functional consequences in T cells. First, rafts may concentrate the α chains (CD25) in the vicinity of signaling IL-2R β and γ_c chains, forming a common signaling platform in the membrane, before cytokine stimulation. This 'focusing' effect may enhance the association rate of the high affinity receptor upon IL-2 binding, even if IL-2Rα does not bind directly IL-2 [49,50].

This property may partly be responsible for the increased proliferation rate of leukemia/lymphoma T cells compared with normal peripheral T cells. Consistent with the present data, the recently observed 'preassembly' of IL-2R subunits [11] in leukemia/lymphoma T cells may be brought about by sorting a fraction of overexpressed α chains together with the constitutively expressed β and γ_c chains to common membrane microdomains via 'raft-mediated trafficking'. Compartmentation of the IL-2R chains by rafts in these cells may also assist in setting up the proper conformation of heterotrimer IL-2R and its association with further intracellular (or raft-associated) signaling molecules to gain full signaling capacity. This is possibly brought about by a conformation-dependent tightening of their interactions upon binding of the relevant cytokine [11].

Second, the GPI microdomains can also concentrate other cytokine receptor α chains (e.g. IL-4Rα IL-7Rα or IL-15Rα) in the locality of common γ chains shared by them for signaling [2,6]. This hypothesis, however, requires further investigation with the above mentioned α chains.

Third, co-compartmentation of CD25 with CD48 may further provide a regulatory platform for GPI-anchored proteins in T cell physiology and growth. Recent work, reporting on inhibition of T cell growth but not of effector function upon immobilization of GPI-anchored proteins CD48, Thy1 or Ly6A/E by cross-linking with antibodies [51], is consistent with this hypothesis. The GPI-microdomains, which are rich in src-family kinases [13,14] may also promote/regulate assembly of the IL-2R subunits with these enzymes in case of signal pathways mediating activation-induced T cell death or survival [2].

The impact of raft-assisted membrane compartmentation on T cell growth signaling was demonstrated by the remarkably reduced IL-2 stimulated STAT3/STAT5 phosphorylation upon disruption of raft integrity. This effect may be brought about by the lateral dispersion of IL-2R subunits resulting in decoupling of the intracellular interaction (crosstalk) between Jaks associated to the β and γ_c chains, respectively. These interactions are known to be essential in the formation of docking sites for downstream signaling molecules, such as STATs, during signal transduction [2].

In conclusion, the present data are consistent with a model where a substantial fraction of IL-2Rα (CD25), together with the constitutively expressed β and γ_c chains, is associated with cholesterol- and glycosphingolipid-rich membrane microdomains (rafts) in cell lines of human adult T cell lymphoma/leukemia origin, independently of the ligand occupation level of IL-2 receptors. These microdomains contain, among others, a potential regulatory protein of T cell growth, CD48. A pivotal role of cholesterol in maintaining such transient protein assemblies, including also HLA glycoproteins, was also demonstrated. Thus, IL-2R chains may represent a new example of the few transmembrane proteins found associated with lipid rafts [14]. It is still unclear which structural motifs result in targeting these polypeptide chains to rafts, as no report has so far been published regarding their acylation (palmitoylation), which is known to promote association with rafts [14,35]. Perhaps their relatively heavy N- or O-linked glycosylation makes them attractive for rafts through potential carbohydrate–carbohydrate interactions with GPI-anchored proteins as well as with the glycosylated headgroups of glycosphingolipids occurring at high density in rafts.

All these properties may be characteristic of human T cell lines of leukemia/lymphoma origin, as a recent study in mouse cell lines [52] revealed a distinct role of lipid rafts in regulating IL-2 signaling, namely sequestration of CD25 by lipid rafts impeding interaction with the IL-2Rβ and γ chains. The observed difference between these and our results focuses attention on the constitutive or induced raft-association of the IL-2R subunits and therefore the regulatory role of lipid rafts in IL-2R signaling may be cell- or species-specific. This is further emphasized by another recent study [53] that reports on raft-association of the IL-2Rβ chain in transformed human NK and fibroblast cells. Thus, to better understand the role of lipid rafts in cell growth/viability-signaling of T cells, in general, and in the unregulated growth of leukemic T cells, in particular, similar comparative investigations seem necessary using mouse vs. human T cell lines and antigen-stimulated T cells from peripheral blood vs. uncultured cells isolated from T cell leukemia. The importance of this question is underlined by the recently reported progress in the IL-2 receptor-targeted immunotherapy of human leukemia/lymphoma [9].

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