Evidence for Chemokine-mediated Coalescence of Preformed Flotillin Hetero-oligomers in Human T-cells*

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Background: Coexpression of the membrane microdomain-associated proteins flotillin-1 and -2 is required for their location in uropods of chemokine-stimulated T-cells.

Results: Coexpression of untagged flotillin-2 in T-cells markedly enhances FRET between flotillin-1-EGFP and flotillin-1-mCherry.

Conclusion: Our data strongly suggest predominant formation of flotillin hetero-oligomers in T-cells.

Significance: This study enhances understanding of the molecular organization of T-cell uropod membrane microdomains.

We have shown previously that endogenous flotillin-1 and -2, closely related proteins implicated in scaffolding of membrane microdomains, are rapidly recruited to the uropods of chemoattractant-stimulated human neutrophils and T-cells and are involved in cell polarization. Coexpressed flotillin-1 and -2, but not singly expressed proteins, are also targeted to the uropod of T-cells and neutrophils. Biochemical studies suggest formation of flotillin homo- and hetero-oligomers in other cell types, but so far knowledge is lacking on in situ flotillin organization in leukocytes. We have now analyzed flotillin organization in human T-cells using fluorescence resonance energy transfer (FRET). Coexpressed C-terminally tagged flotillin-1-mCherry and flotillin-2-enhanced green fluorescent protein (EGFP) show significant FRET when analyzed in intact human T-cells in the absence and presence of chemokine. In contrast, little FRET was observed between coexpressed flotillin-1-mCherry and flotillin-1-EGFP before or after chemokine addition, indicating predominant formation of heterodimers and/or -oligomers. Interestingly coexpression of untagged flotillin-2 strongly enhanced FRET between differently tagged flotillin-1 molecules in resting and chemokine-stimulated cells, indicating that close contacts of flotillin-1 molecules only occur in flotillin-2-containing hetero-oligomers. Comparable results were obtained for tagged flotillin-2. We further show that disruption of the actin network, depletion of intracellular calcium, and inhibition of phospholipase C all result in suppression of chemokine-induced polarization and flotillin cap formation, but do not abolish FRET between tagged flotillin-1 and -2. Our results support predominant formation of flotillin-1 and -2 hetero-oligomers in resting and chemokine-stimulated human T-cells which may importantly contribute to structuring of the uropod.

Chemotactic migration of the highly motile T-cells is indispensable for fulfillment of their functions such as rapid recognition of foreign antigens, stimulation of antibody production, and destruction of virally infected cells or tumor cells. T-cell polarization and directional migration is a complex, not yet well understood process. Certainly it involves a functional cytoskeleton, reversible actin polymerization in the front, and myosin-dependent contractility in the rear of a migrating, polarized cell (1, 2). Polarization of leukocytes requires segregation and activation of specific signaling and cytoskeletal molecules in the retracting rear (uropod) and motile forward moving part (front) of the cells. Localized positive feedback loops and inhibitory effects of front signaling pathways on rear signaling and vice versa are thought to reinforce this structural and biochemical polarization (3). Plasma membrane microdomains (“rafts”) have been implicated to stabilize polarity of migrating leukocytes (4).

We have studied reorganization of reggie/flotillins, putative raft-scaffolding proteins, in polarizing leukocytes. Flotillin-1 and -2 are two highly homologous proteins whose enrichment in membrane microdomains has been ubiquitously observed. Flotillins are thought to be involved in structuring membrane microdomains and have been implicated in the delivery of membranes and membrane proteins to cell contact sites, regenerating axons, growth cones, etc. (5). We have recently shown that flotillin-1 and -2 interact and, upon stimulation with chemotactic agents, rapidly form membrane caps and at later time points accumulate in the uropods of polarized primary human neutrophils and T-lymphocytes. Capping of endogenous flotillins in human neutrophils and T-cells depends on actin dynamics but does not require Rho-kinase or myosin II activity. Capping of ectopically expressed flotillins requires cotransfection of flotillin-1 and -2, suggesting that uropod expression requires heterodimer or hetero-oligomer formation (6, 7). However, data on the organization of flotillins in T-cells, and the impact of cell stimulation on oligomerization, are not yet available. Transfection of T-cells with a dominant negative mutant of flotillin-2 impaired cell polarization and uropod capping of endogenous flotillin-1 and the adhesion receptor P-selectin glycoprotein-1, indicating a functional role of flotillins in cell polarization (7). These findings are in line with those obtained by Ludwig et al. (8), who studied neutrophils from flotillin-1 knock-out mice. Their data showed that the lack of flotillin-1, which leads to a reduction of flotillin-2 and its displacement.
from rafts, results in impairment of murine neutrophil uropod formation and migration through Matrigel (8).

Biochemical studies in HeLa cells and Neuro2a cells, using cross-linking and sucrose gradient centrifugation of cell lysates, suggest that endogenous as well as ectopically expressed flotillins form homo- and hetero-oligomers (9–13). Evidence for direct interactions of flotillin-2 with flotillin-2 and -1 have also been obtained using the yeast two-hybrid system (9, 10). According to Frick et al. (12), overexpressed flotillin-1 and -2 assemble in a 1:1 ratio to produce microdomains in HeLa cells. C-terminal regions (as 184–321) in flotillin-2 have been implicated in oligomerization (11). Babuke et al. (13) provided data using FRET in HeLa cells suggesting that cell stimulation with EGF induces clustering of preexisting flotillin oligomers.

Most of these data have been obtained with in vitro or invasive methods involving cell solubilization, in cells of low motility. We have now studied in situ organization of the flotillin scaffolds in human T-cells before and after addition of chemokine using FRET assays. We provide evidence for significant FRET between C-terminally tagged flotillin-2-EGFP and flotillin-1-mCherry in resting and chemokine-stimulated human T-cells, which is not abolished by treatments that suppress T-cell polarity and flotillin cap formation such as disruption of F-actin, depletion of cytosolic calcium, or inhibition of phospholipase C (PLC). We also present evidence strongly suggesting the predominant presence of hetero-oligomeric flotillin complexes in resting and chemokine-stimulated human T-cells.

EXPERIMENTAL PROCEDURES

Materials and Suppliers—Stromal cell-derived factor 1 (SDF-1) was from Peprotech. Latrunculin A, U73122 were from Alexis Biochemicals. Bovine serum albumin (BSA) was from Serva. Gey’s solution contained 138 mM NaCl, 6 mM KCl, 100 μM EGTA, 1 mM Na2HPO4, 5 mM NaHCO3, 5.5 mM glucose, and 20 mM Hepes (pH 7.4), without or with 1 mM MgSO4. Antibodies against CD14, CD16, CD19, CD36, CD56, CD123, and CD235a (glycophorin A). Cells binding these antibodies were subsequently depleted from the cell suspension using anti-biotin MicroBeads. The resulting cell suspension contained >95% T-lymphocytes as assessed using anti-CD3 staining. The cells were used after overnight incubation in RPMI with 10% FCS at 37 °C and 5% CO2.

Immunostaining of Flotillin-1 and -2—T-cells were incubated as described in the figure or table legends, followed by fixation with TCA and staining for flotillin-1 and -2, as described (7).

Transient Transfections of T-lymphocytes—For transfections, 3–6 × 10^6 freshly isolated T-lymphocytes were resuspended in 100 μl of human T cell nucleofector solution (Amaxa) diluted 1:2 with PBS, and 1.5 μg of plasmid DNA per vector was added. Then, the cell suspension was transferred to a cuvette, and nucleofection was carried out (Amaxa Nucleofector, program U-14). Immediately, 500 μl of medium with 20% FCS was added, and the cells were transferred to a prewarmed 12-well plate containing 2.5 ml of medium with 20% FCS, followed by incubation at 37 °C in a CO2 incubator for 6 h. Transfected cells were subsequently washed, resuspended in Gey’s solution, and used for experiments.

FRET—Transfected cells were treated without or with inhibitors and SDF-1, as described in the figure legends, followed by fixation with paraformaldehyde (PFA) as described (7). Imaging was performed on fixed samples with a confocal laser scanning microscope Olympus FluoView FV1000-IX81, 60× oil immersion objective, using the acceptor photobleaching module. The acceptor (mCherry) signal was bleached in defined regions of interest (ROIs) with 559 nm light at 100% laser power to ~15% of the initial acceptor fluorescence intensity. The change in donor (EGFP) fluorescence induced by acceptor photobleaching was quantified by comparing prebleach and postbleach images obtained by excitation at 473 nm (0.5% laser power). FRET efficiency was calculated as (I_{post} − I_{pre})/I_{post} × 100 where I_{post} is the intensity of EGFP before the bleach and I_{post} is the intensity of EGFP after the bleach in defined ROIs. The background was determined by outlining a ROI in a region containing no cells. This background value was subtracted from each value obtained in the cells. The mean FRET intensities obtained in at least 30 ROIs from at least three different transfections were measured for each protein pair and cell treatment.

RESULTS

Colocalization of Endogenous and Ectopically Expressed Flotillins in Freshly Isolated Human T-cells—Endogenous flotillin-1 and -2 showed marked colocalization in resting and chemokine-stimulated freshly isolated human T-cells (Fig. 1A), comparable with data obtained for human expanded T-lymphoblasts (7). Resting cells were mainly spherical, with a punc-
tate, membrane-associated location of both flotillin-1 and -2, and occasional flotillin caps. Upon stimulation of cells with the chemokine SDF-1 for 15 min, the majority of the cells polarized, correlating with exclusive location of both flotillins at the plasma membrane at the tips of the uropods (Fig. 1A and Table 1). All of these data are comparable with our previous findings for human T-lymphoblasts (7). Ectopically expressed flotillin-1-mCherry and flotillin-2-EGFP showed comparable locations and extensive colocalization in resting and stimulated T-cells and did not modify chemokine-induced shape changes (Fig. 1B). As shown previously in T-lymphoblasts (7), singly expressed flotillins did not cap in the uropod of freshly isolated T-cells (see below). A possible explanation is that there are not enough free endogenous uncomplexed flotillins available for hetero-oligomer formation between tagged singly expressed flotillins and endogenous flotillins. Our previous results suggested that formation of flotillin heterodimers or hetero-oligomers occurs in T-cells and that this process is a prerequisite for uropod targeting (7). The data shown in Fig. 1 also indicate that the C-terminal tags do not disturb the normal location of flotillins. As flotillin heterodimer formation appears to be a functionally important process, we applied FRET to study its occurrence and regulation in human freshly isolated T-cells.

**Interactions of Flotillin-1 and -2 in T-cells Studied with FRET—** We studied *in situ* interactions of ectopically expressed flotillin-1-mCherry and flotillin-2-EGFP with a FRET approach in fixed cells. As the C-terminally tagged flotillins assume the same location as endogenous flotillins in T-cells (Fig. 1), they are well suited for this technique. According to Albertazzi et al. (14), EGFP and mCherry show a good spectral overlap and

**FIGURE 1. Colocalization of endogenous and ectopically expressed flotillin-1 and -2 in human T-cells.** A, T-cells were preincubated for 30 min at 37 °C followed by a further incubation for 15 min without or with 40 ng/ml SDF-1, fixation with trichloroacetic acid, and staining for endogenous flotillin-1 (flo1) and flotillin-2 (flo2). B, T-cells were cotransfected with flotillin-1-mCherry and flotillin-2-EGFP followed by incubation as described in A, and PFA fixation. Scale bar, 10 μm.

**TABLE 1**

**Impact of calcium depletion or phospholipase C inhibition on T-cell polarization and flotillin capping**

For the analysis of the impact of calcium depletion, T-cells were preincubated in Gre’s buffer lacking calcium but containing 1 mM MgSO4 and without or with 6.1 mM EGTA for 30 min at 37 °C, followed by a further incubation for 15 min without or with 40 ng/ml SDF-1. For the analysis of the impact of phospholipase C inhibition, cells were preincubated for 30 min at 37 °C in Gre’s buffer with calcium and magnesium containing either 0.1 μM inactive control substance U73343 or 0.1 μM phospholipase C inhibitor U73122, followed by a further incubation for 15 min with 40 ng/ml SDF-1. Cells were then fixed with TCA and stained for flotillin-1 (Flo1) and flotillin-2 (Flo2). The fractions of polarized cells and of cells with flotillin caps were determined for 100 cells/sample. Mean ± S.E. of three independent experiments are shown.

| Treatment | Polarized cells | Flo1 caps | Flo2 caps |
|-----------|----------------|-----------|-----------|
| Buffer    | 7 ± 3          | 9 ± 5     | 8 ± 3     |
| SDF-1     | 85 ± 9         | 85 ± 10   | 87 ± 8    |
| EGTA/SDF-1| 1 ± 1          | 1 ± 1     | 1 ± 1     |
| Buffer    | 14 ± 9         | 11 ± 6    | 14 ± 8    |
| SDF-1     | 89 ± 14        | 92 ± 5    | 97 ± 2    |
| U73343/SDF-1| 91 ± 6       | 89 ± 7    | 96 ± 3    |
| U73122/SDF-1| 1 ± 1        | 2 ± 1     | 2 ± 2     |
allow reproducible quantification of energy transfer. A Förster radius of 51 Å for FRET was determined for these two fluorescent proteins (14). We used bleaching of the acceptor mCherry in fixed cells, which results in an increase in donor (EGFP) fluorescence in case of close proximity of the two fluorescent protein tags, for FRET determinations. For resting cells, part of the membrane-associated flotillins were bleached; for the SDF-1-stimulated cells, part of the uropod cap was bleached. Substantial FRET efficiency (15 ± 4%, 50 ROIs) was obtained for coexpressed flotillin-1-mCherry and flotillin-2-EGFP already in the absence of chemokine, which was increased (p < 0.0001) to 25 ± 3% (120 ROIs), by chemokine stimulation of T-cells (Figs. 2, A and B, and 3B). We explain this small increase in FRET induced by stimulation of cells by SDF-1 by accumulation of more binary FRET pairs within the analyzed area due to coalescence of preexisting oligomers in caps rather than by changes in the extent of oligomerization.

As controls we also studied FRET in cells transfected with the tags alone, that is, mCherry and EGFP. Here we obtained a value of FRET efficiency slightly above background values obtained outside of cells, that is, 1 ± 1% (Fig. 3A). In cells transfected with flotillin-2-EGFP and flotillin-1-EGFP, apparent FRET efficiency also was very low (2 ± 3%, Fig. 3A).

We showed previously that disruption of the F-actin network by latrunculin A completely prevents SDF-1-induced formation of endogenous flotillin uropod caps in T-lymphoblasts, whereas the caps are resistant to inhibition of Rho-kinase or myosin II (7). We obtained comparable results for capping of endogenous flotillins and ectopically expressed flotillins in freshly isolated human T-cells (data not shown and Fig. 2C for latrunculin A). Moreover, we previously showed, using fluorescence recovery after photobleaching in T-lymphoblasts, that flotillins are partially immobilized already in resting cells, and that almost complete immobilization occurs upon stimulation of cells with SDF-1. Mobility of flotillins is increased markedly in resting and chemokine-stimulated cells by latrunculin A, indicating immobilization of flotillins by F-actin (7). We now wanted to know whether F-actin also modulates flotillin oligomerization. Treatment of T-cells with latrunculin A prior to addition of SDF-1 completely suppressed cap formation and prevented the small chemokine-induced apparent FRET increase (Figs. 2C and 3C) without, however, affecting FRET in resting cells (Fig. 3F).

We further observed that incubation of T-cells with 6.1 mM EGTA (minimal concentration required for full effects) to deplete intracellular calcium completely disrupts SDF-1-induced formation of endogenous flotillin caps and suppresses cell polarity (Table 1). Inhibition of PLC, which induces formation of inositol 3-phosphate and a subsequent increase in intracellular calcium, by U73122, but not the inactive control substance U73343, also completely suppressed endogenous flotillin cap formation and cell polarization, even in the presence of millimolar extracellular calcium, confirming a requirement for intracellular calcium transients for these events (Table 1). We then studied the impact of calcium depletion or PLC inhibitors on FRET between flotillin-1-mCherry and flotillin-2-EGFP in SDF-1-stimulated T-cells. Calcium depletion as well as PLC inhibition prevented the chemokine-induced small apparent FRET increase (Figs. 2, D–F, and 3, D and E). EGTA or PLC inhibition did not further reduce FRET in resting cells (Fig. 3F).

In summary, treatments that prevent flotillin cap formation do not affect flotillin-1 and -2 FRET in resting cells, indicating that they do not modify hetero-oligomer formation.

Cotransfection with Untagged Flotillin-2 Markedly Enhances FRET between Flotillin-1-mCherry and Flotillin-1-EGFP in Human T-cells—According to the literature, flotillins form homo- as well as heterodimers (9–13). We now assessed possible formation of flotillin homodimers in resting and stimulated T-cells. As shown in Fig. 4, A and B, coexpressed flotillin-1-mCherry and flotillin-1-EGFP do not form caps dependent on stimulation with SDF-1, but rather show a linear plasma membrane association both in resting and chemokine-stimulated cells. They also show a low FRET value in the absence of SDF-1 of 6 ± 2% (30 ROIs), which was not increased by SDF-1 (Figs. 4, A and B, and 5A). Cotransfection of untagged flotillin-2 with flotillin-1-mCherry and flotillin-1-EGFP induced SDF-1-dependent capping of tagged flotillin-1 and interestingly marked FRET between flotillin-1-mCherry and flotillin-1-EGFP. In resting cells values of 23 ± 2% (60 ROIs) and in SDF-1-stimulated cells values of 26 ± 3% were obtained (Figs. 4, C and D, and 5A). Our data strongly suggest that flotillin-1 molecules are only brought into close proximity by interacting with flotillin-2 molecules, indicating predominant formation of flotillin hetero-oligomers in resting and activated T-cells. Comparable results were obtained for flotillin-2-mCherry and flotillin-2-EGFP expressed in the absence or presence of untagged flotillin-1 (Fig. 5B).

DISCUSSION

Previous data strongly but indirectly suggest that flotillin heterodimer or hetero-oligomer formation is a prerequisite for uropod targeting in neutrophils and T-cells and that flotillins importantly contribute to leukocyte uropod structuring (6–8). Biochemical data, using the yeast two-hybrid system, or cross-linking, or coimmunoprecipitation, or size analysis of flotillin complexes in cell lysates, suggest formation of both homo- and hetero-oligomers in other cell types (9–13). Most of these data were obtained in HeLa cells, cells with low motility. However, the detergent used for solubilization of cellular flotillins in these studies may modify flotillin interactions. Moreover, solubilization of flotillin complexes, which reside at least partly in detergent-insoluble fractions, may be incomplete. We now have used in situ FRET to directly study flotillin organization in human T-cells, circumventing these problems. We observed substantial FRET between the randomly membrane-associated flotillin-1 and -2 already in resting cells. SDF-1 stimulation induced a small FRET increase accompanied by coalescence of the flotillin molecules into a sharply defined membrane-associated cap at the tip of the uropod (Figs. 2 and 4). These data strongly suggest that flotillin heterodimers or hetero-oligomers are formed already in resting cells, giving rise to small mobile membrane-associated platforms, corresponding to the punctate appearance of flotillins in resting cells (Fig. 1). Coalescence of flotillin platforms in stimulated cells is accompanied by an apparent increase in FRET, which is probably mainly due to coalescence of preexisting oligomers into a smaller area,
FIGURE 2. FRET between flotillin-1-mCherry and flotillin-2-EGFP in human T-cells: impact of chemokine, disruption of F-actin, depletion of calcium, or inhibition of PLC. T-cells were transfected with flotillin-1-mCherry (flo1-mCherry) and flotillin-2-EGFP (flo2-EGFP) and treated as follows. A and B, cells were preincubated for 30 min at 37 °C followed by a further incubation for 15 min without (A) or with 40 ng/ml SDF-1 (B). C, cells were preincubated for 30 min at 37 °C with 1 μM latrunculin A followed by a further incubation for 15 min with 40 ng/ml SDF-1. D, cells were preincubated for 30 min at 37 °C in Gey's buffer lacking calcium, with 1 mM MgSO4 and 6.1 mM EGTA, followed by a further incubation for 15 min with 40 ng/ml SDF-1. E and F, cells were preincubated for 30 min at 37 °C in Gey's buffer with calcium and magnesium containing either 0.1 μM inactive control substance U73343 (E) or 0.1 μM PLC inhibitor U73122, followed by a further incubation for 15 min with 40 ng/ml SDF-1 (F). FRET images were acquired in cells fixed with PFA after the treatments indicated above. A–F, images show EGFP and mCherry fluorescence before (left columns) and after (middle columns) acceptor (mCherry) photobleaching. Right columns, top show FRET efficiency; bottom show differential interference contrast images of cells. ROIs used for determination of FRET by acceptor photobleaching are outlined in white in the middle lower panels. Scale bars, 5 μm.
although a small increase in FRET pair formation cannot be excluded. These data are comparable with those obtained in HeLa cells (13).

The question arises how flotillin hetero-oligomerization is regulated. As shown previously, flotillin capping in T-cells and neutrophils is independent of Rho-kinase or myosin II activity but requires actin dynamics (6, 7). Prevention of actin polymerization suppresses flotillin capping and prevents immobilization. We now observed that suppression of actin polymerization does not affect FRET between flotillin-1 and -2 in resting cells and conclude that hetero-oligomerization occurs independently of a direct or indirect actin interaction of flotillins.

We have now also studied the role of calcium and PLC in regulating T-cell polarization as well as flotillin capping and hetero-oligomerization. PLC isoforms are important downstream targets of chemokine-mediated signaling in T-lymphocytes. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate to produce inositol 3-phosphate and diacylglycerol. Inositol 3-phosphate then induces the release of calcium from intracellular stores. Depletion of intracellular calcium stores results in activation of calcium release-activated calcium channels resulting in calcium influx into the cells, whereas diacylglycerol activates protein kinase C (PKC) isoforms. Both PLC activity and increases in cytosolic calcium...
have been implicated in T-cell migration (15, 16). In none of these studies has the role of calcium and PLC in T-cell polarization and flotillin capping been addressed. We now show that both calcium depletion and PLC inhibition completely prevent SDF-1-induced T-cell polarization and flotillin capping (Table 1). In resting cells, flotillin FRET was not affected by calcium depletion or PLC inhibition (Fig. 3). Heterooligomerization is thus not regulated by levels of intracellular calcium or PLC activity.

To obtain further insight into organization of T-cell flotillin membrane microdomains, we investigated whether homodimers/oligomers also are formed. Homodimer formation has been suggested by data obtained for flotillin-2 with the yeast two-hybrid system (9, 10). However, little FRET was observed between coexpressed flotillin-1-mCherry and flotillin-1-EGFP or flotillin-2-mCherry and flotillin-2-EGFP in our work (Figs. 4 and 5). Interestingly, marked FRET of these tagged proteins, in the range of that obtained for coexpressed flotillin-1-mCherry and flotillin-2-EGFP, was induced by coexpression of untagged flotillin-2 or -1, respectively. The presence of flotillin-2 in the flotillin oligomers is thus essential for FRET between flotillin-1 molecules, and vice versa. The interaction with flotillin-2 in these complexes brings two flotillin-1 molecules within distance for substantial FRET to occur (<70 Å). These data strongly suggest almost exclusive formation of flotillin hetero-oligomers in both resting and chemokine-stimulated T-cells (Figs. 4 and 5). We cannot, however, completely exclude that flotil-
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FIGURE 5. FRET between flotillin-1-mCherry and flotillin-1-EGFP or flotillin-2-mCherry and flotillin-2-EGFP in human T-cells is significantly enhanced by cotransfection with untagged flotillin-1: quantitative evaluation of data. T-cells were cotransfected with flotillin-1-mCherry (flo1-EGFP) and flotillin-1-EGFP (flo1-EGFP) (A) or flotillin-2-mCherry (flo2-mCherry) and flotillin-2-EGFP (flo2-EGFP) (B) in the absence or presence of untagged flotillin-2 (flo-2) (A) or flotillin-1 (flo1) (B), as indicated. Cells were incubated in the absence or presence of SDF-1 as described in the legend of Fig. 4 and fixed with PFA. FRET images were acquired as described in the legend of Fig. 2. Data are represented as FRET efficiency (percentage) (mean ± S.D.) determined in 30–70 ROIs derived from 3–7 independent experiments for each condition. Significance of differences between data was calculated using the Kruskal-Wallis test with Dunn’s multiple comparison test. **, p < 0.01; ***, p < 0.001.

Lins also form homodimers in T-cells, but that the fluorescent tags are in an angle unfavorable for FRET in these complexes. We would then have to postulate a conformational change induced by hetero-oligomerization that now allows closer interaction and as a consequence FRET between differently tagged flotillin-1 or flotillin-2 molecules. This is, however, rather unlikely based on the proposed dimensions of the C-terminal flotillin oligomerization domains (approximately 5 × 1.5 nm) (17) compared with that of EGFP and mCherry (approximately 3 × 4 nm) (18).

Solis et al. (11) studied flotillin oligomerization in Neuro2a cells using a membrane-permeable cross-linker that links adjacent lysine residues within a range of ~11 Å. After cross-linking, cell lysates were analyzed with immunoblotting. 200-kDa bands could be detected corresponding to homotetramers and/or heterotetramers of the endogenous flotillins. Solis et al. (11) also analyzed oligomerization of exogenously expressed proteins (EGFP-tagged flotillin-2 and HA-tagged flotillin-1) using cross-linking, which allows one to distinguish formation of homo- and hetero-oligomers. Here, comparable formation of flotillin-1 and -2 homotetramers and heterotetramers was observed, somewhat in contrast to our findings in T-cells. Possibly, flotillin oligomerization is regulated in a cell type-specific manner. Flotillins contain a domain in their C terminus that is predicted to form three adjacent coiled-coils. Coiled-coil 2 (as 239–321) and parallel coiled-coil 1 (as 184–238) domains in flotillin-2 may mediate tetramerization. Homodimeric or heterodimeric proteins, possibly formed by a four-stranded parallel coiled-coil, have been suggested to be the only stable state of flotillins (11). According to our work, this would be predominantly flotillin hetero-oligomers, at least in human T-cells.

In summary, we provide evidence for almost exclusive formation of hetero-oligomers of flotillin-1 and -2 in resting and chemokine-stimulated human T-cells, whose formation is independent of an intact F-actin network and is not regulated by PLC or intracellular calcium. These hetero-oligomers cluster upon cell activation and may importantly contribute to the structuring of the T-cell uropod. Open questions concern the size of the hetero-oligomers, the precise arrangement of flotillins in the hetero-oligomers, and the possible regulation of oligomerization by posttranslational modifications.

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