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Minireview

A Role for Lipid Rafts in Immune Cell Signaling

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***Abstract*: Cross-linking of surface receptors in hematopoietic cells results in the enrichment of these receptors in the rafts along with other downstream signaling molecules. A possible explanation how signal is transduced through the plasma membrane has arisen from the concept of raft. From the study of cellular responses in the plasma membrane which enrich members of the Src-family tyrosine kinase, rafts can function as centers of signal transduction by forming patches. Under physiological conditions, these elements synergize to transduce successfully a signal at the plasma membrane. Rafts are suggested to be important in controlling appropriate protein interactions in hematopoietic cells, and aggregation of rafts following receptor ligation may be a general mechanism for promoting immune cell signaling.**

***Key words*: Raft, Glycosphingolipid, B cell antigen receptor, Fcε receptor, T cell receptor, Src-family tyrosine kinase**

Introduction

Since Singer and Nicolson proposed the fluid-mosaic model (27), the cell membrane has been thought to be a two-dimensional liquid crystal, where membrane proteins are uniformly solubilized in lipid solvent. This simple view has been questioned by several studies. Simons and Ikonen (26) postulated the existence of rafts, lateral assemblies of glycosphingolipids (GSL) and cholesterol, which associate with specific proteins. Rafts are viewed as platforms to serve as scaffolds to facilitate apical sorting or the association of the signaling molecules, increase the rate of interactions, and enhance crosstalk networks. These functionally distinct microdomains are also called glycolipid-enriched microdomain (GEM), detergent-insoluble microdomain (DIM), detergent-resistant microdomain (DRM), low density microdomain (LDM), Triton-insoluble floating fraction (TIFF) etc., based on their insolubility in non-ionic detergents under certain conditions, e.g., 1% Triton at 4 C and buoyant density on sucrose density gradients.

Although the idea of rafts to serve as signaling domain was quite appealing, there has been controversy in their existence in living cells. Detergents used might cause artifacts during isolation, but this possibility can be excluded since rafts could be obtained with detergent-free methods (28, 29). The biophysical studies confirm them in artificial lipid reconstitution systems (1). Recent studies (17, 18, 31) using fluorescence resonance energy transfer (FRET) microscopy reached opposite conclusions about the existence of rafts in cell membranes of living cells. However, another technique using newly discovered property of green fluorescence protein (GFP) represented that although the clustering caused by glycosylphosphatidylinositol (GPI) anchors was significant, it must be rather loose and/or transient (3). Besides the microscopic studies, chemical cross-linking with

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Abbreviations: BCR, B cell antigen receptor; BL, Burkitt's lymphoma; CT-B, cholera toxin B subunit; DIM, detergent-insoluble microdomain; DRM, detergent-resistant microdomain; FcεRI, Fcε receptor I; FRET, fluorescence resonance energy transfer; Gb3, globotriaosylceramide Galα1→4Galβ1→4Glcβ1→1 ceramide; GC, germinal center; GEM, glycolipid-enriched microdomain; GFP, green fluorescence protein; GM1, Galβ1→3GalNAcβ1→4NeuAc(α2→3)Galβ1→4 Glcβ1→1 ceramide; GPI, glycosylphosphatidylinositol; GSL, glycosphingolipid; HA, hemagglutinin; HEL, hen egg lysozyme; IIPLC, class II peptide loading compartment; ITAM, immunoreceptor tyrosine-based activation motifs; LAT, linker for activation of T cell; LDM, low density microdomain; MAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MIRR, multichain immune recognition receptors; SMAC, supramolecular activation clusters; Stx, Shiga toxin; TCR, T cell receptor; TIFF, Triton-insoluble floating fraction.

bifunctional reagents was used to show that GPI-anchored proteins are in spatial vicinity on the cell surface (7). These data suggest that rafts are rather small (of about 70 nm in diameter) and that they are very dynamic.

The basic principle of raft function is to accumulate or segregate different proteins in lateral domains of the membranes. Rafts as signaling domains have been particularly well documented for immune reaction where a series of molecules involved in triggering have been suggested to become associated to raft domains upon stimulation of the receptor. Antigen receptors, co-stimulatory molecules, Src-family tyrosine kinases and GPI-linked proteins show dynamic movement within the cell membrane during lymphocyte differentiation and signaling.

In this review, we describe the overview of rafts and the role of them from immunological aspects with the recent reports including our study on human renal cell line and Burkitt's lymphoma cells (BL).

Biochemical Properties of Rafts

The key biochemical characteristics used to study the composition and function of rafts are insolubility in the detergent Triton X-100 at 4 C and buoyancy. Rafts are recovered as low density microdomains from detergent lysates after sucrose density gradient centrifugation (20). Figure 1 gives an instance of separation of lipids from ACHN, human kidney cancer cell line (16).

A variety of signaling molecules have been reported to reside in rafts, including proteins interacting either with the outer or with the inner leaflets of the plasma membrane (Table 1). Many of these proteins are modified by the addition of saturated lipid groups, which prefer to more ordered environment of lipid rafts (11). These modifications are done by the addition of a GPI anchor or myristate N-acyl or palmitate S-acyl groups, resulting in the insertion of modified proteins into raft membranes. Some (e.g., Ras) are acylated with prenyl groups, containing unsaturated bonds and/or branch. Association of these proteins with rafts is easily disrupted by non-ionic detergents. Lipid modification is not always required for proteins inserted into rafts. Influenza hemagglutinin (HA) is a well-studied raft associated transmembrane protein. Mutations of the HA polypeptide in the transmembrane region that faces the exoplasmic leaflet abrogated the Triton X-100 insolubility of HA and thus influenced its raft association (25). Conversely, lipid modification does not always target proteins to rafts. Some lipid-modified proteins such as the transferrin receptor (10) are acylated at multiple sites but are excluded from rafts.

Cholesterol functions to keep GSL assemble tightly and effectively. Treatment with fillipin, an antibiotic which specifically binds to cholesterol, dramatically

Table 1. Lipids and signaling proteins constitutively associated with rafts

Lipids	GM1 cholera toxin receptor
	GM3 cell adhesion
	GD1b associates wth FcεRI
	GD3 TAG-1 signaling
	Gb3 Stx receptor
	sphingomyelin
	cholesterol
Proteins	GPI-anchored proteins
	Src-family kinase
	GTP-binding proteins
	Receptor for T/B Ag, Fcε, EGF, PDGF etc.
	LAT (linker for activation of T cells)
	NO synthetase

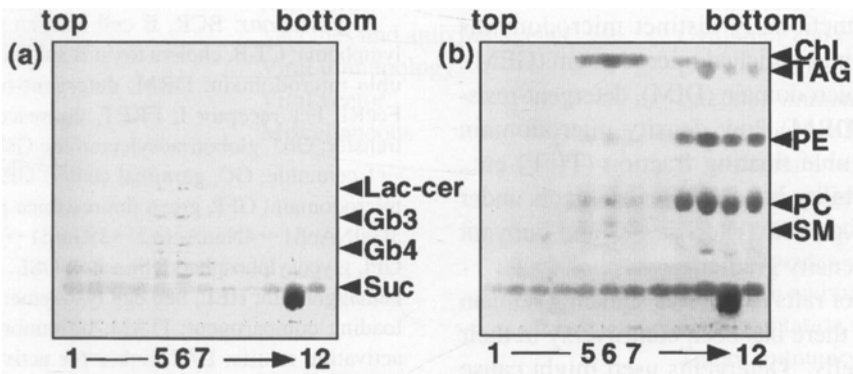

Fig. 1. TLC analysis of lipid in fractions separated by sucrose density gradient centrifugation. TLC plates were sprayed with Orcinol-H_2SO_4 reagent and Dragendorf-Dittmer reagent to detect GSL (a) and phospholipids (b), respectively. Chl, cholesterol; TAG, triacylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

reduces association of Src-family kinase Yes with rafts and abrogates Yes activation by Stx binding to globotriaosylceramide (Gb3) (16). Cholesterol depletion with cyclodextrin causes a reduction in components of the mitogen-activated protein kinase (MAPK) cascade associated with rafts and hyperactivation of the remaining Erk in the fraction. This suggests that the MAP kinase pathway can connect the cholesterol level of rafts to the control of cell division (8).

Hypothetical model of the lipid organization and associated molecules is shown in Fig. 2.

Rafts in Lymphoid Cells

It is now clear that signal initiation by antigen-mediated clustering of all multichain immune recognition receptors (MIRR) requires tyrosine phosphorylation at specific sequences in their cytoplasmic segments, the immunoreceptor tyrosine-based activation motifs (ITAM), by Src-family tyrosine kinases that are constitutively present at the cytoplasmic side of the plasma membrane. Since many parts of Src-family kinases were found in rafts, rafts have been thought to play an integral role in coupling MIRR to Src-family tyrosine kinases as functional microdomains.

1. Fcε Receptor I on Mast Cells

Fcε receptor I (FcεRI) is the high affinity receptor for IgE on mast cells and basophils. The Src-family tyrosine kinase Lyn is known to associate with and participate in the signaling mediated by aggregation of FcεRI (4). The stoichiometry and affinity of Lyn association with FcεRI are low in lysates from unstimulated cells, but antigen-mediated aggregation of IgE receptor complexes causes an increased association of active Lyn. Baird and her co-workers (5, 6, 13) showed that cross-linking of IgE-FcεRI complexes on mast cells caused their association with rafts in a cholesterol-dependent process and that Lyn associated with rafts is responsible for antigen-mediated tyrosine phosphorylation of FcεRI β and γ chain. They carefully chose the lower concentration of Triton X-100 and the higher concentration of EDTA for lysis buffer as compared with those of previous reports by others. The lysis buffer they used enabled them to detect distinct differences in IgE-FcεRI distribution before and after cross-linking of IgE-FcεRI complexes and to minimize the nonspecific bindings of proteins to rafts. The temporal aggregation of raft components and tyrosine phosphorylation of FcεRI are prolonged under disruption of actin polymerization, indicating that association of FcεRI with Lyn is ceased by segregating motility of actin filament. They postulated that interaction between Lyn and cross-linked IgE-FcεRI is regulated by stimulated F-actin polymerization, and this is best explained by a segregation of anchored raft components from more mobile components. This study provides new insights

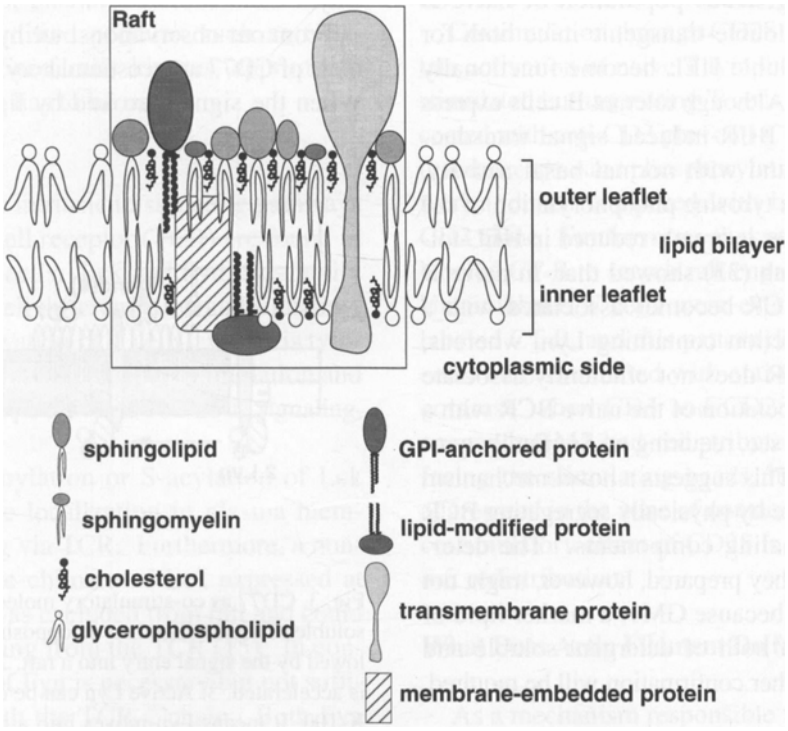

Fig. 2. Hypothetical model of the lipid organization.

into the structural dynamics of rafts in cells and their roles in signal transduction by FcεRI.

2. B Cell Antigen Receptor

The B cell antigen receptor (BCR) serves both to initiate signal transduction cascades and to target antigen for processing and presentation by MHC class II molecules. Both the signaling function and the nature of surface Ig transmembrane region appear to specify the correct targeting of the BCR to the class II peptide loading compartment (IIPLC) and to accelerate the rate at which the antigen is targeted to the IIPLC. Since rafts are not static entities *in situ* but instead dynamic microdomains on the cell surface to which proteins and lipids have variable affinities, it is highly probable for rafts to play an important role in BCR signaling and antigen targeting. Cheng et al (2) provided evidence for a role for rafts in the initial steps of BCR signaling and antigen targeting. Upon cross-linking, BCR rapidly translocates into GM1-enriched lipid rafts that contain Src-family kinase Lyn and exclude phosphatase CD45R. Both Igα and Lyn in the rafts become phosphorylated, and subsequently BCR and a portion of GM1 are targeted to the IIPLC. Entry into rafts, however, is not sufficient for targeting to the antigen processing compartments.

3. Tolerant B Cells

How the BCR is selectively uncoupled from some signaling pathways is unclear. Mice expressing transgenes for anti-hen egg lysozyme (HEL) IgM/IgD BCR contain a large homogeneous population of naive B cells. The B cells in double-transgenic mice both for anti-HEL BCR and soluble HEL become functionally tolerant (anergic) (22). Although tolerant B cells express proteins important for BCR-induced signal transduction at normal levels and with normal basal activity, antigen-induced protein tyrosine phosphorylation of the BCR and other molecules is greatly reduced in HEL-tolerant B cells. Weintraub (33) showed that, in naive B cells, the stimulated BCR becomes associated with a detergent-insoluble fraction containing Lyn, whereas, in tolerant B cells, BCR does not efficiently associate with this fraction. Association of the naive BCR with a raft occurs in less than 6 sec, requiring no Src-family tyrosine kinase activation. This suggests a novel mechanism of tolerance maintenance by physically segregating BCR from downstream signaling components. The detergent-insoluble fraction they prepared, however, might not be equal to a lipid raft, because GM1, a marker lipid of rafts, was recovered in both of detergent soluble and insoluble fractions. Further confirmation will be required.

4. CD 77$^+$ Germinal Center B Cells

The germinal center (GC) of secondary lymphoid follicles is the site where B cells are destined to be deleted or spared, namely, "clonal deletion" or "clonal selection," respectively. Antigen binding to BCR as well as other co-stimulatory signals is believed to cooperate in regulating this process. CD77, i.e., Gb3, expressed on a fraction of GC B cells is a candidate regulator of GC B cell apoptosis (21). Since BL cells are thought to correspond to CD77-positive GC B cells, it can be used as an *in vitro* model of GC B cell apoptosis in which CD77 is involved. CD77 has also been known to serve as a receptor for Shiga toxin (Stx), an enterotoxin produced by several strains of *Escherichia coli*. Cells expressing CD77, including BL lines, are sensitive to Stx and readily undergo apoptosis. Using Stx-sensitive BL Ramos cells as an *in vitro* model of CD77$^+$ GC B cells, we examined intracellular signaling events mediated by either Stx or anti-CD77 antibody (19, 24). We observed that Stx binding to CD77 in rafts induced recruitment and activation of Lyn, accompanied by hyperphosphorylation of Syk as well as an increase in complex formation between Lyn, Syk and μ chain. Also observed is a synergism between cross-linking of CD77 and that of surface IgM in the apoptosis-inducing effect in BL cells. Furthermore, multiple caspases, including caspase-3, -7 and -8 were promptly activated following Stx1 treatment. These results raise a possibility that CD77 participates in GC B cells apoptosis by affecting the BCR-signaling cascade via a raft-mediated signaling pathway.

From our observations, we hypothesized the involvement of CD77 as a costimulatory molecule in raft (Fig. 3). When the signals evoked by ligands-binding to CD77

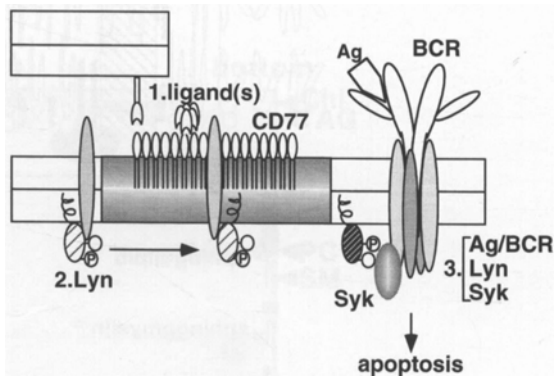

Fig. 3. CD77 as co-stimulatory molecule for BCR. 1: Ligand(s), soluble or immobilized on an opposing cell, binds to CD77, followed by the signal entry into a raft. 2: Lyn moves into a raft and is accelerated. 3: Active Lyn can be easily solubilized by Triton X-100. It means Lyn moves into a non-raft region, where Lyn associates with both BCR and Syk. See in text.

transmits in raft, resting or inactivated Lyn previously located outside moves into raft and then the activation of Lyn is accelerated. Some spanning molecules may serve Lyn for entering a raft and for activation. Detergent solubility of activated Lyn seemed to be increased, and by this effect, activated Lyn leaves raft for a 'non-raft' region, where Lyn becomes associated with both of BCR and Syk, another protein tyrosine kinase. This association results in affecting of BCR state, or lowering of threshold of BCR and enhancing tyrosine phosphorylation of Syk, followed by cell death, i.e., clonal deletion.

The seconds-ordered phosphorylation of FcεRI by Lyn in mast cells was not due to the elevation of Lyn's specific activity, but to a spatial change of activated Lyn (5), whereas the minutes-ordered phosphorylation of Syk by Lyn in GC B cells was due to the sequential change of specific activity and distribution of Lyn. Thus, our study is an example describing the participation of rafts in BCR-signaling cascade and suggests a possible role of CD77 as a regulator of BCR-induced apoptosis in human B cells.

Localization of immune receptors to rafts has yielded conflicting results. One possible reason for these discrepancies may come from the difference in the stringency of detergent extraction. Some studies used 1% Triton X-100, whereas some used 0.02% Triton X-100 or 1% Brij, a weaker detergent than Triton X-100. In such experiments, attention should be given whether proteins recovered in rafts are contaminated or not. Since immune receptors may be anchored to rafts more weakly than other lipid-modified proteins such as GPI-anchored proteins and Src-family kinases, appropriate stringency of detergents has to be used.

5. T Cell Receptor

A number of proteins intrinsic to signaling pathways upon ligation of the T cell receptor (TCR) are found in rafts of unstimulated T cells. Lck and Fyn, Src-family kinases essential for T cell differentiation and signaling, and LAT (linker for activation of T cells), which is tyrosine phosphorylated following receptor stimulation and is required for both PLC-γ1/Ca^{2+} and Ras/ERK signaling, are well studied.

Inhibition of myristoylation or S-acylation of Lck suppressed not only the localization in plasma membrane, but also signaling via TCR. Furthermore, a non-acylated transmembrane chimera of Lck expressed at the plasma membrane was excluded from raft and could not fully support signaling from the TCR (15). In contrast to Lck, acylation of Fyn is necessary but not sufficient for interaction with the TCR ζ chain. Both Fyn kinase and SH_2 domains were required, directing phosphorylation of ζ ITAM tyrosines and subsequent binding of the Fyn SH_2 domain to ζ ITAM phosphotyrosines (30). The transmembrane adapter protein LAT is also S-acylated and is present in rafts, but the non-acylated mutant form of LAT is excluded from rafts although it remains attached to the plasma membrane segment. The LAT mutant fails to become tyrosine phosphorylated after TCR stimulation and is incapable of activating the PLC-γ1/Ca^{2+} and Ras/ERK pathways (34). Above results suggest that not only targeting to the plasma membrane but also the localization within the plane of the plasma membrane are equally crucial for these signaling molecules to fulfill their function. A similar relationship between function in signaling and localization to membrane domains exists for Ras proteins.

A complementary experimental approach is to visualize rafts in intact cells by staining with fluorescently-labeled cholera toxin B (CT-B) subunit. CT-B specifically binds to the raft-associated glycosphingolipid GM1 and can be cross-linked with anti-CT-B antibody to form membrane patches (14). Aggregation of the TCR by anti-CD3 monoclonal antibody (mAb) cross-linking also caused coaggregation of raft-associated proteins. However, CD45 did not colocalize to either CT-B or CD3 patches. Cross-linking of either CD3 or CT-B strongly induced tyrosine phosphorylation and recruitment of a ZAP 70$(SH)_2$-GFP fusion protein to the lipid patches. These results suggest a mechanism whereby TCR activation involves the aggregation of rafts and segregation of Lck and CD45, mediated by their differential affinity for these structures, which stimulates PTK signaling.

Costimulation through CD28 is well known to facilitate activation of naive T lymphocytes. Viola et al (32) stimulated human resting T cells with polystyrene beads coated with anti-CD3 plus anti-CD28 mAb and observed that more tyrosine phosphorylation on multiple proteins were induced compared with beads coated with anti-CD3 alone. Furthermore, they stained GM1 with FITC-labeled CT-B to search raft engagement in this process. Unstimulated T cells were evenly stained with FITC-labeled CT-B, and this pattern did not change when the cells were stimulated with anti-CD3 coated beads. By contrast, when CD3 and CD28 were simultaneously engaged, GM1 had redistributed to form a dense cap facing the stimulating beads, indicating that rafts had aggregated in the zone of contact. This suggests that the costimulatory effect of CD28 is achieved by its role for raft redistribution.

What Does Actin Filament Do for Raft Redistribution?

As a mechanism responsible for raft redistribution, a reorganization of actin filament after cross-linking has

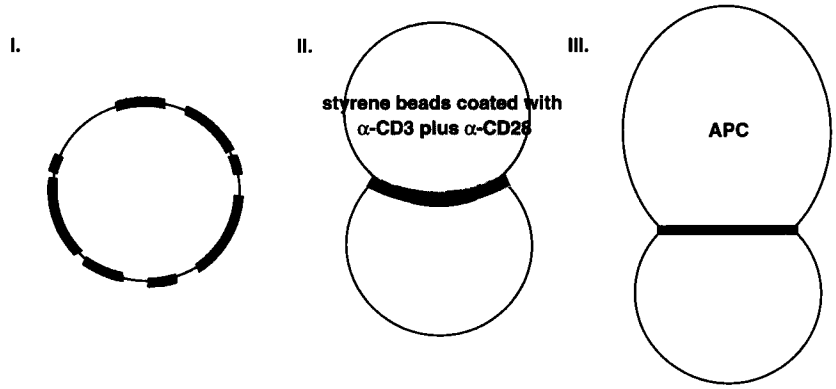

Fig. 4. The changes of membrane domains induced by three different ways. I: Cross-linking with soluble form of antibodies. A few–several μm of patches appears. II: Intensive and simultaneous stimuli both from Ag receptors and co-stimulatory molecules. A dense cap formation occurs. III: Stimulation by a professional APC. SMAC is formed at a contact plane between a T cell and an APC. Ag receptor complex accompanying with signaling molecules aggregate in cSMAC, as well as adhesion molecules keeping two cells contact strictly accumulate in pSMAC. Cytoskeleton are elongated from this area.

been postulated to participate. Harder and Simons (12) studied cellular responses to raft patch formation focusing on particular changes in the actin cytoskeleton. They found that filamentous actin accumulates in raft patches formed by cross-linking of GPI-anchored protein and GM1 and that this process requires phosphotyrosine accumulation in patches. Tyrosine phosphorylation of patch proteins, however, does not require accumulation of actin cytoskeleton. These observations show a link between raft-mediated signaling and the interaction of actin cytoskeleton with raft membrane domains. Viola et al (32) described in its footnotes that stimulation of T cells with anti-CD3 plus anti-CD28 induced a reorganization of actin filament.

Monks et al (23) analyzed three-dimensional distribution of receptors and intracellular proteins that cluster at the contact between T cell and APC during antigen-specific interaction using a digital imaging system. Receptors and intracellular proteins involved in physiological T cell activation are organized into distinct spatial domains, named supramolecular activation clusters (SMAC). TCR/CD3 complex, Lck, Fyn and PKCθ accumulated in the central SMAC (cSMAC), whereas LFA-1 and talin localized in the peripheral SMAC (pSMAC). Actin filaments is elongated from talin linking to LFA-1 beneath the membrane. It is highly probable that actin cytoskeleton is involved in dense cap formation in T cells stimulated with anti-CD3 plus anti-CD28 mAb as well as in raft redistribution. Although patch formation and activation of Src-family kinases occurred within a few minutes after cross-linking, dense cap formation or TCR/CD3 complex localization in cSMAC appeared over 10–60 min. Quick activation of Src-family kinase by ligand binding may not require the aid of actin cytoskeleton, but cytoskeleton may serve to help raft patches persist after cross-linking. In the case of FcεRI signaling, actin filaments contribute to the segregation of raft components and tyrosine phosphorylation of FcεRIs. Thus, cytoskeleton may play bifunctional roles either in patch stabilization or in disaggregation depending on cell type. Raft redistribution, dense cap formation with anti-CD3 plus anti-CD28 and SMAC formation between T cell and APC are schematically drawn in Fig. 4.

General Remarks

The involvement of raft in immune signaling suggests an important physiological role for these membrane domains in immune cell activation. However, the molecular mechanisms of immune receptors translocated to rafts remain to be elucidated. The rapidity of the translocation was common to all immune reactions. Presumably, a specific phosphorylation in Igα/Igβ within ITAMs is insufficient to enter into the rafts in order to initiate significant downstream signaling. Furthermore, it is not clear whether raft redistribution reflect physiological stimulation of lymphoid cells. The recent interesting reports on SMAC and the immunological synapse (9) show segregation of signaling molecules within contact sites. This stable architecture probably overcomes the problems of low TCR-ligand affinity and low ligand concentration. In future studies, it will therefore be important to confirm the presence of raft aggregation at immunological synapse and to determine the molecular mechanism which drives raft organization in this context.

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