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T cell synapse assembly: proteins, motors and the underlying cell biology

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Abstract

A tantalizing feature of the 'immunological synapse' is the segregation of transmembrane proteins into activating clusters and their underlying signalosomes. The mechanisms by which transmembrane proteins are initially recruited to and then stably segregated at the synapse remains an outstanding question in the field; and one likely to reveal key modes of signaling regulation. Ongoing real-time imaging approaches and a refocusing of efforts upon understanding the basic cell biology of T cells have all contributed to a developing model of T cell behavior; elementary TCR-derived signaling quickly feeds back into the basic cellular programs controlling cell shape, adhesiveness, motility, as well as some poorly understood aspects of membrane fluidity and segregation. It is increasingly clear that the mechanisms for control at this level are shared between T cells and other cell types and may not be revealed in differential genomic screening. To this end, imaging-based genetic screens are now coming online to aid in identifying the ubiquitous proteins that function at polarized signaling surfaces.

Keywords: T cell polarity; Immunological synapse; Integral membrane proteins; Microscopy; Imaged-based screening

1. Introduction

Microscopy-based studies of molecular dynamics in the immune response have undergone an explosion in interest. This has been most dramatic in the analysis of T cell signaling at the antigen-presenting cell (APC) contact face, an interface now known as the 'immunological synapse' (IS) [1]. Kupfer and coworkers first used the then-novel technique of deconvolution microscopy together with antibody staining of fixed T cell/APC couples to demonstrate the supramolecular organization of the contact face. Here it was found that molecules at the interface are arrayed with T cell receptors and MHC molecules occupying a central zone of the interface (the *c*entral-Supra*m*olecular *A*ctivating *C*luster or c-SMAC) and the integrin-ligand pair LFA-1-ICAM occupying the periphery (*p*eripheral-SMAC) of this contact [2]. Subsequent studies have demonstrated the diversity of the IS, pointing out that in some cases, SMACs never completely coalesce or are replaced by multifocal synapses [3,4]

Confocal and widefield real-time imaging approaches have been enhanced for live-cell observations and have dramatically extended our understanding of these molecular assemblies. In an elegant system, Dustin and colleagues imaged unlabeled T cells interacting with a supported lipid bilayer in which fluorescent ligands were seeded. This method has proven particularly desirable since a single optical *z*-section contains the bulk of the receptor–ligand interaction [1,5]. In addition, precise calibration of the relationship between fluorescent intensity and molar concentration of ligands seeded into the bilayer permits kinetic parameters to be determined [5]. More recent advances in video rate acquisition (e.g. cameras based on high-photosensitivity, high data-rate CCD chip) promise to further enable this approach since higher speeds allow the characterization of molecular dynamics for systems whose rates of diffusion or supramolecular aggregation might otherwise exceed the interval frame rate.

The use of green-fluorescent protein (GFP)-tagged receptors in live T cells interacting with live APCs required the additional adoption of fast piezzo-electric objective-based *z*-motors [6,7]. This is because the contact surface is not known

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prior to the start of the experiment for T cells undergoing activation with a moving 3-dimensional surface (i.e. a live APC)—sometimes T cells form contacts head on, sometimes they 'scan' up and over APCs as part of their activation program leading to an ever-changing synapse location. Use of a spinning-disk confocal head further improves the approach by the associated improvement in resolution in the *x–y* axis [7].

This fast-3D approach has had added benefits—namely that events at rapidly evolving contact sites can be captured in the context of APCs bearing a complete cohort of ligands while also generating spatial density data for the entire cell surface and not just the reaction face. Using such an approach has permitted a complete characterization of three-dimensional diffusion and transport rates within labeled cells [8], a result that further emphasizes that 'free diffusion' alone cannot account for the molecular behavior. This increased field-of-view has permitted the observation and quantification of signaling-related events more traditionally associated with cell-biology, including changes in cell shape, cell velocity and the overall distribution of proteins within the membrane, front to back.

Here we review some of the fundamental aspects of this latter feature—the redistribution of proteins within the membrane during signaling onset and their organized assembly at the IS. Starting with the features of transmembrane proteins that make this a site of control, we describe some of the membrane-based events in motile cells that are likely subject of regulation. From this, we address the potential role of myosin motor proteins in regulating membrane protein distribution. Finally, we outline a next-generation of genome wide analysis that uses the 3D localization criterion to identify gene-products that assemble at particular cellular locations during a relevant signaling process.

2. Cell surface molecular movement during the crawling-synapse transition

An oft-overlooked feature of T cells is that they are inherently highly polarized cells, although it is not often apparent for naive cells ex vivo in the absence of chemokines. T cells in their native lymph nodes in vivo or when pre-activated and observed in vitro take on an amoeboid form, perhaps as a default but more likely as a result of G-protein-coupled receptors (GPCRs) responding to local and polarized chemotactic stimuli. For these cells, a leading edge is defined by pseudopodal projections in the direction of migration while the trailing edge is constricted to form a 'tail' or 'uropod'. The nucleus is contained in the leading edge bulb while the uropod contains much of the free cytoplasm and the microtubule organizing center (MTOC).

Such a pre-synapse phenotype is clearly a result of cooperative cellular processes. In particular, crawling is an interplay between actin polymerization at the leading edge, depolymerization at the trailing edge, and tensioning of intermediate zones, probably via Class II myosin motors. Some of the best models for T cell motility come from the studies of *Dictyostelium*. In both T cells and *Dictyostelium*, protrusion at the leading edge as well as the formation of a uropod require a functional actin cytoskeleton [9,10]. While actin-based protrusion occurs in the absence of Class II myosin motors [11], both leading edge and trailing edge dynamics require functional actin. A working model for cytoskeletal control of T cell polarity can also be extrapolated from studies in migrating neutrophils. Here, Rac activation of actin polymerization in the leading edge induces protrusion while Rho activity in the tail appears to limit protrusive actin and potentially facilitate depolymerization [12]. Antagonism between Rac and Rho is likely to reinforce the distinction between leading edge and trailing edge actin behavior and thus represent a self-reinforcing feedback loop [12]. The mechanism for the control of tensioning via Myosin II is not completely clear but it would appear that more dramatic Myosin light-chain kinase activity is correlated with the trailing edge in neutrophils, thus providing greater tension in the highly pinched uropod. It is clear that this motor is generally enriched in the uropod and at the junction of the uropod-leading edge. Loss of myosin or myosin ATPase activity in T cells results in a loss of the uropod and an inability to translate leading-edge pseudopodal protrusions into cell movement [11]. Based on the sum of this evidence, we have suggested that the class II myosin in the uropod therefore acts in a protrusive manner—essentially squeezing the trailing edge cellular-content into the space created by actin nucleation at the leading edge [11].

Curiously, the leading edge is dramatically more sensitive to TCR-mediated signaling than the uropodal projection [13]. This effect is not due to any particular costimulatory activity of APCs, as T cells show this preference when challenged with either peptide pulsed B cells or polystyrene beads coated with anti-CD3 [13,14]. This highly active leading edge then becomes the location of the IS and polarity obviously prevails throughout the contact—c-SMAC and p-SMAC distributions are highly biased molecular aggregations at the former leading edge.

A paradoxical but quickly obvious feature of TCR/CD3 distribution is the predominance of these molecules in the uropod in the pre-activation crawling T cell form. As shown in Fig. 1A, the uropodal membrane is disproportionally enriched in TCRs. Given the final form of the synapse, it is obvious that a TCR-loaded synapse requires a large number of molecules to translate rear to front. Indeed, when studied in real-time approaches, this initial rear-bias is observed to be rapidly corrected when agonist stimuli are received at the leading edge—at speeds of approximately 0.2 μm/min consistent with motor processivity [8]. As shown in the example in Fig. 1B, within 90 s of the onset of calcium signaling, the leading edge (now the synapse-contact face) is enriched for CD3 molecules whereas the uropodal pool has largely disappeared. That this is due to movement on the cell surface is shown by the use of anti-TCRβ-FAb-coated beads

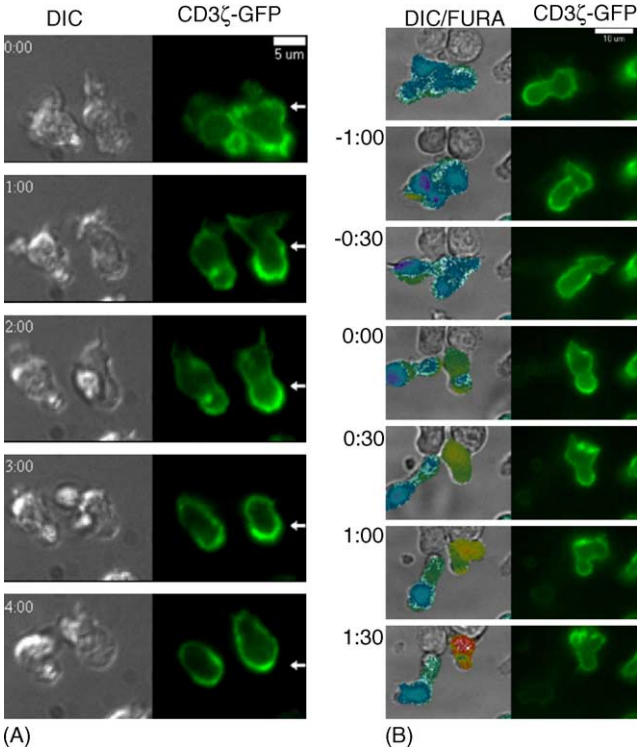


Fig. 1. Pre-polarization and rapid repolarization of TCR/CD3 chains to the immunological synapse. (A) Distribution of CD3zGFP in D10 T cells during normal crawling behavior. (B) Recruitment (within 90 s) of CD3zGFP to the immature IS on encountering a B cell loaded with 10 μM CA 134–146, an agonist peptide for the D10 TCR.

which also move smoothly and directionally toward the contact face [8]. Indeed, older studies using avidin beads bound to surface-biotinylated T cells or intercalated into the lipid bilayer showed that much more than the TCR by itself is moved during this process. Indeed a bulk pool of NHS-biotin accessible and/or lipid-labeled contents undergoes flow towards the synapse location [15] as do a number of specific receptors (e.g. CD4) and even non-specific ones such as the Transferrin receptor [16]. Clearly, a great deal of the lipid bilayer and its contents may undergo some bulk flow towards the leading edge but the specificity for this is not known. While recent reports have suggested an internalization at the rear followed by secretion at the leading edge as an alternative means to synapse assembly [17], the bead data strongly argues for surface movement as the primary mechanism, at least for the pre-existing cell-membrane associated pool of receptors. Notably, analysis of the forward-directed velocities of beads as compared to analysis of the total 3D pool are both in good agreement and all of the observed beads traffic along a path confined to the surface, further supporting that surface movement largely accounts for the redistribution of the total pre-existing membrane associated pool. It is, of course, possible that some cell types with higher basal levels or rates of TCR internalization will use the secretion mechanism more heavily.

3. The politics of transmembrane proteins: overall mechanisms of localization and the lipid Raft hypothesis

What then defines the localization of the T cell receptor prior to and during T cell engagement? One emerging model suggests that membrane ‘fences’ and ‘posts’ [18,19], perhaps comprising the spectrin/ankyrin cytoskeleton [20], might create ‘corrals’ within the lipid bilayer and thus restrict movement in the membrane. This concept is discussed in some detail in the article by Kosumi in this issue and other reviews by Edidin and coworkers [20] and will therefore not be repeated here. In the context of synapse assembly, however, such mechanisms would typically act to restrict supramolecular assembly during the fluid phase leading up to the c-SMAC. Existing models supply no clear motive force for selecting the proteins to aggregate within a given corral or otherwise promote aggregation. Furthermore, features of a living cell such as retrograde flow (discussed below in connection with myosin II) and the directional redistribution of proteins to the synapse are likely to require a more active process.

A pragmatic assessment of integral transmembrane proteins suggests three general regions to which positional determinants for membrane movement can be mapped (diagrammed in Fig. 2). In the type ‘A’ protein–protein interactions, the extracellular domain can interact with either ligands on adjacent cells, or with adjacent proteins within the lipid bilayer. In this way, forces may be applied to the protein ectodomain and aid in assembling one protein together with others or relative to a fixed fiducial along the cell surface. Similarly, in type ‘B’ interactions, the intracellular cytoplasmic amino acids can engage in protein–protein interaction directly with intracellular cytoskeletal elements or indirectly with such elements via linker proteins. As in the type ‘A’ interactions, here protein–protein interactions would ultimately be used to move proteins to new locations. Both type ‘A’ and ‘B’ interactions are easily subject to regulation in response to conformational changes and/or the presence of binding partners in the respective cellular region. Ultimately, both type ‘A’ and ‘B’ interactions may be used in concert with a regulated ‘fence’ lattice to confine complexes, once they are formed. Indeed, these types of interactions are the most commonly cited ones in models for supramolecular assembly and cellular movement.

Perhaps the most exciting concept for segregation of transmembrane proteins is the direct interaction of the protein with the lipid bilayer itself and translational movement associated with segregation of components of the lipid bilayer. Despite much effort, this is a highly undeveloped area and one whose importance is almost certainly at the heart of the segregation problem. Associated with this idea is the observation that the lipid bilayer is composed of many component lipid building blocks and that certain proteins might thereby be preferentially ‘solvated’ by particular types of lipids [21]. The component lipids are numerous and vary from cytoplasmic membranes to intracellular membranes and even from baso-

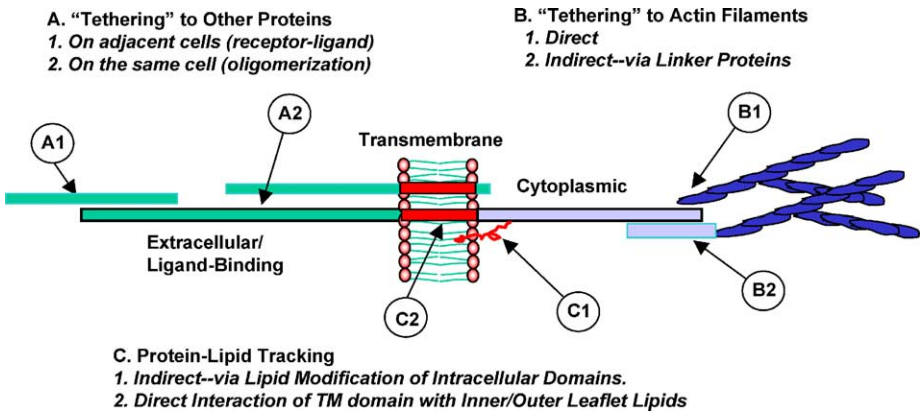


Fig. 2. Potential interactions driving membrane protein localization. A model integral transmembrane receptor is shown. Sites at which interactions might occur, thereby influencing movement and/or position are grouped into three classes, based on type of interaction.

lateral to apical surfaces [22]. FRET-based studies have lent strong support to the intuitive concept that these lipids are not freely miscible and that some domains (e.g. GPI-containing) self-aggregate in the plasma membrane of cultured cells, excluding other lipid components and perhaps their associated proteins [23].

The interactions of a transmembrane protein with particular components of the lipid bilayer, (its 'solvation' in a lipid environment), can be composed of two types of interactions. As denoted 'C1' in Fig. 2, lipid modification is sometimes found on the cytoplasmic domains of transmembrane and on fully cytoplasmic proteins. These modifications in turn aid in the membrane attachment of these proteins, as well as potentially segregating them to particular lipid environments. As examples, it is well established that signaling proteins such as *lck*, LAT and RAS are variously myristylated, palmitylated, and farnesylated as a means to increase their membrane association. In the cases of those molecules, the acylation of the protein is associated with segregation of these proteins into detergent insoluble biochemical fractions, called lipid 'rafts' (also known as DIGs or GEMs) [24,25]. Such modifications are also occasionally present on transmembrane proteins themselves, for example the palmitoylation site on CD4 [26].

A prevailing model of lipid 'rafts' suggests that segregation of proteins with these lipid linkages via preferential association with large islands of specific lipids in the membrane may contribute to transient confinement of these together. This is, of course, particularly appealing as it would provide for a designated chemical microenvironment in which signaling might be precisely controlled by inclusion/exclusion of key enzymes. However, it is becoming likely that the current biochemical definitions of raft association (e.g. low-density and detergent insoluble fractions following Triton X-100 cell lysis [24]) and the physical reality of a lipid-protein interaction that influences membrane mobility and localization may ultimately not prove to be complementary. Many studies examining the 'lipid environment' of a protein fail to differentiate between the intracellular and extracellular pool of these proteins and thus confound analysis. More importantly, many of these results are highly subject to experimental conditions, for example, the effects of the critical micelle concentration of detergents used. Perhaps most confounding is that domains beyond the transmembrane insertion point might ultimately influence solubility in detergent—a lysis approach inherently introduces the possibility of creating fractions where no similar fractions existed in vivo [27].

Despite strong feelings regarding the validity of 'raft' isolation and analysis, it remains important not to throw the baby out with the bathwater. It remains clear that solvation of proteins in specific components of the lipid bilayer will affect both the proteins' position on the surface as well as its lateral mobility. If lipid segregation occurs, then regardless of the type A and B interactions that move the protein, the transmbembrane domain will be forced to reside in a specific lipid environment. Is this interaction neutral or is there specificity?

4. The politics of transmembrane proteins: the role of transmembrane domains?

The role of the transmembrane domain in influencing lateral positioning is unclear. Its role in selecting a lipid microenvironment and perhaps protein–protein associations has largely been ignored. Traditionally, transmembrane domains have been considered somewhat amorphous regions of proteins, consisting of and defined by small aliphatic and lipophilic amino acids that are modeled to readily form an alpha helical domain. However, the details of such domains are far from consistent and the work of many groups, most notably Engelman's has revealed a good deal of structure in these regions of proteins.

For the purposes of this review, we assembled a quick survey of some of the best-characterized transmembrane proteins in T cells. This reveals that such domains can be casually defined based on two clearly variable features: the total length of their hydrophobic domains and the hydrophobicity

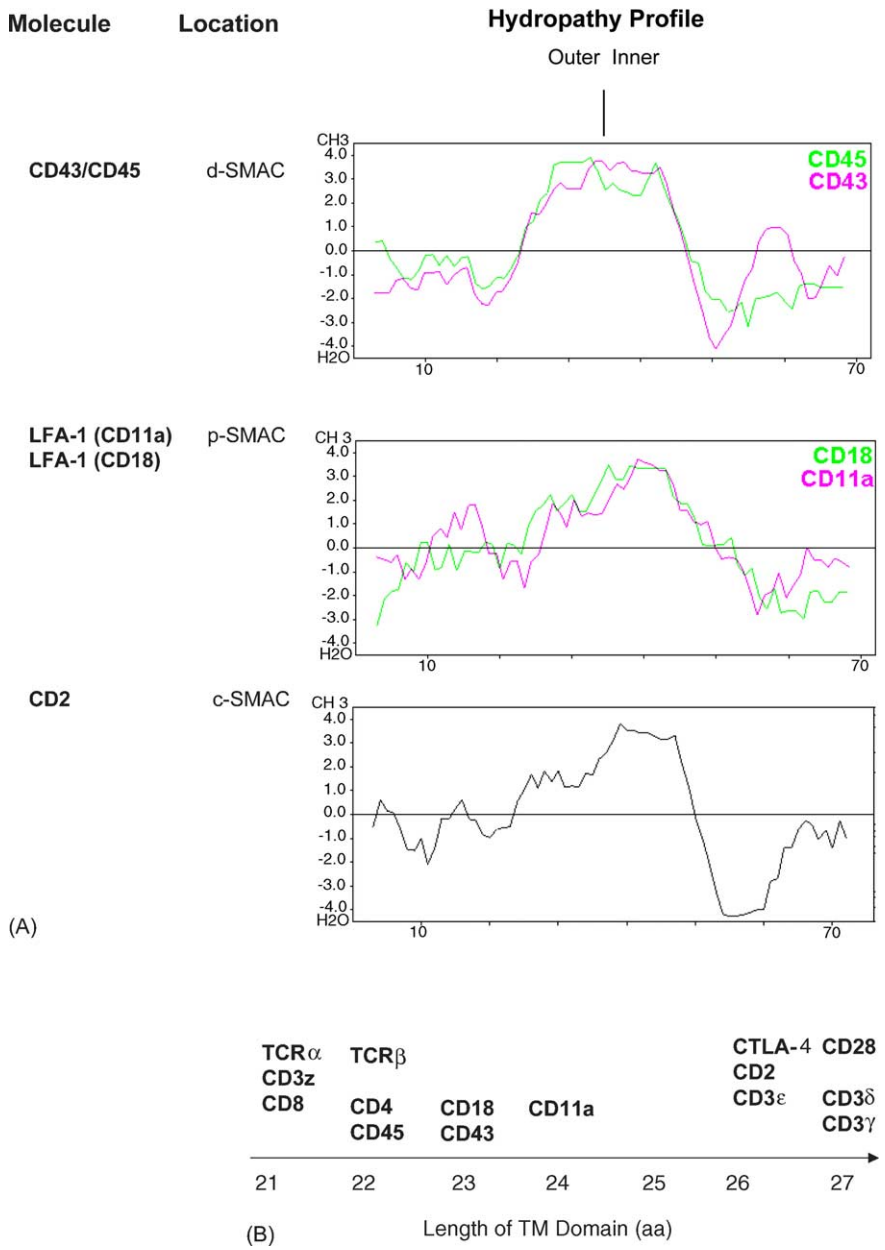


Fig. 3. Diverse nature of transmembrane domains of some known T cell surface proteins. The Genbank sequence of the mouse polypeptide encoding each of the indicated proteins was used to extract the putative transmembrane domain plus 25 N- and C-terminal amino acids. (A) These were subject to GREASE analysis (Kyte-Doolitle) using a window of seven peptides. The resulting plots are shown grouped into two classes, based upon total length of the TM domain as well as the hydropathy in the regions putatively assigned as 'outer' or 'inner' leaflet (by dividing the total TM domain into two equal halves). (B) Annotations for predicted TM lengths based on such analysis were collected for T cell surface receptors and plotted according to the predicted length of the transmembrane domains.

profile in the amino acids corresponding to the outer leaflet of the lipid bilayer (see Fig. 3). Doubtless, there is a great deal more subtlety than is revealed in the figure here—however, it is worth emphasizing the point that transmembrane regions are quite diverse in their biophysical features and prediction of their interactions with specific lipid components or with one another in the lipid context is a developing science.

Molecules such as CD43 and CD45 are grouped together in Fig. 3A on the basis of shorter (22–23 aa) and consistently very hydrophobic TM domains from the putative inner-leaflet to the outer-leaflet regions, containing only strongly hydrophobic aliphatic residues and no glycine residues. In contrast, many of the ligand-bound receptors such as CD2, LFA1, (also CD28, CTLA-4) to name a few, have somewhat longer hydrophobic domains (∼24–28 aa) containing conserved glycine residues in the outer leaflet region.

At the level of primary amino acid sequence, the importance of hydrophobicity and amino acid composition is likely twofold. First, the variation in hydropathy indicated by the glycine rich region of the outer leaflet may represent a fa-

vorable interaction between proteins or with particular lipid constituents of the membrane. To this extent, proteins with a relatively lower hydropathy index in the outer leaflet domains may preferentially be solvated by cholesterol (or some other lipid) and thus subject to a lower-fluidity environment. While, as of this writing, the importance of such disparities is unclear, it is interesting that outer-leaflet amino acids of the haemagglutinin protein, HA, have been shown to influence the ability of this protein to biochemically segregate into DIGs, perhaps indicating the importance of this domain in determining lipid solvation characteristics [28] (with the numerous caveats associated with 'raft isolation' as well as conflicting reports suggesting palmitoylation as the critical raft-determinant [29]). Second, weak helix-helix pairing between adjacent proteins in a bilayer is observed in a number of transmembrane proteins, including signaling proteins such as the EGF receptor. Characterization of transmembrane motifs by Engelman and co-workers has suggested that at least one glycine containing motif within TM domains, GxxxG, can encourage helix-helix pairing and/or weak multimerization [30]. These investigators have made note of the fact that such weak interactions are relatively promiscuous [30]—indeed in the context of this discussion it might be argued that interactions of the transmembrane domains may represent an elementary (albeit weak) mechanism whereby components of a future signaling complex move together as larger multimers.

The difference in length alone may also have important consequences and in Fig. 3B, we have grouped some of the common T cell surface receptors according to length of TM domain. Along this axis, TM length appears to be mostly well correlated with co-segregation and/or multimerization. For example CD11a and CD18, both components of the LFA-1 complex have similar lengths. Similarly, the TCR α/β heterodimer are of similar TM size and these correspond to the lengths of the coreceptors CD4 and CD8.

It has long been recognized that packing transmembrane domains into a bilayer requires some degree of hydrophobic matching of domains (reviewed in [31]). For example, a longer domain buried within a membrane may be forced to fold with a looser helical pitch around particular lipids within the bilayer domain or contain a subdomain near the bilayer boundary that interacts with a larger lipid which itself sticks out beyond the bounds of the phospholipid bilayer. As an alternative, the membrane itself may have variable girth with shorter transmembrane domains confined to regions separate from those of longer domains. While it is tempting to suggest that 'larger' lipids might determine membrane girth, Engelman and coworkers have addressed this formally and show that integral proteins and not lipids determine membrane girth [22]. Attempts to incorporate longer proteins into a narrow membrane may place the lipid bilayer under tension as it attempts to accommodate the extra hydrophobic region—this would be expected to change the energy of deformation of such a membrane [22]. Alternatively, the increased length may force these to lie at an angle relative to shorter transmembrane domains, thus altering their propensity to self-associate with these shorter domains via intramembrane helix-helix interactions. Any of these possibilities may influence the preferred lipids that solvate the protein and it is noteworthy that length of the TM domain can influence protein sorting, for example from Golgi membranes to cell-surface membranes (reviewed in [32]).

Astute eyes will observe the notable exception in Fig. 3B; namely that TCR heterodimer and CD3ζ contain shorter $\sim$22 aa TM domains while the remaining CD3 chains bearing considerably longer domains. The significance of this may indicate and denote preferential pairing of the groups together (e.g. the $\varepsilon\gamma$ and $\varepsilon\delta$ heterodimers versus the TCRα/β ζ chains), perhaps initially during sorting in the ER and Golgi, but may also indicate a positional requirement for the CD3$\varepsilon\gamma\varepsilon\delta$ transmembrane receptors to lie on an angle relative to the TCR$\alpha\beta$/CD3ζ_2 cluster. This in turn may influence the behavior of the complex when bound versus unbound by peptide MHC complexes.

It remains to be determined what role, if any, these domains will ultimately play in assembly of the synapse components. However, it is intriguing to find that the hydropathy index at numerous amino acids positions as well as TM lengths in the key transmembrane proteins in T cells are highly conserved across evolution, suggesting that this feature may be more important for their function than we previously realized.

5. Integrating molecular events into the cellular framework: the realm of cell-biology

Up to this point, we have focused upon the observed bulk organization of transmembrane receptors in living cells and subsequently upon the biophysical features of the transmembrane domains themselves. The question remains to be determined how these two are tied together. In the particular case of integral membrane proteins within a lipid phase: How does protein–protein aggregation and/or lipid bilayer intercalation give rise to the bulk patterns of receptor distribution observed prior to and following synapse formation? Are there molecules that help microdomains form, move components in/out/away when needed and in other ways contribute toward the stability and function of large-scale membrane-based clusters?

To ask this question is essentially to become a cell biologist—it is integral with considering how mechanisms in the cell function to nurture dynamic events and also to enforce stability. As such, there are probably two good answers. First, one imagines the process to co-opt systems for molecular and organelle movement within an existing cytoskeletal framework. For this, well-known classes of molecular motors move proteins within the cell and particularly along the membrane. To these, we will devote the following section. Second, the process might be facilitated by generalized stabilizing factors at the surface that aid in holding clusters together. At present, these are only hypothesized to exist

(membrane fences and posts may be a subset of these) and the known players are fairly few. For identifying ubiquitous proteins that help stabilize a cellular conformation in protein assemblies at signaling interfaces, a novel form of screening is likely to be required to find the collection of proteins that assemble at these sites, and this screen is considered within the final section.

6. Motor proteins as determinants of membrane topology and protein localization

The role of the cytoskeleton in mediating localization of transmembrane proteins is abundantly clear. Treatment of many cell types with actin depolymerizing or free-actin sequestering drugs uniformly causes the loss of overall polarity and the loss of polar distributions of receptors. In T cells, cytochalasin D treatment leads to a free diffusion of TCRs across the cell surface and an absolute inability to form long-lived TCR microclusters or SMAC assemblies [6].

While cytoskeletal filament remodeling, for example via WASP/Scar type proteins, is undoubtedly important for generating a dynamically changing T cell cytoskeleton, it remains an unlikely candidate for directly moving around proteins. At best, controlled actin nucleation, branching and disassembly can set and re-set boundaries for a synapse, perhaps release key protein components at specific times and possibly support a membrane 'fence'. In some models, this may be sufficient to control the biology. For example, proteins released at the tail and then efficiently tethered at the leading edge would result in profound molecular polarization. This mechanism, sometimes colloquially termed a 'roach motel' mechanism ('molecules come in but never leave') is appealing on grounds of simplicity and molecular independence from cell biological processes. However, analysis of surface bead movement and distribution suggests that: (1) proteins on the surface are carried on a retrograde current, toward the rear pole during crawling—this retrograde current, well described in *Dictyostelium*, utilizes class II myosin motors and the effects of a similar current are seen in the movement of beads toward the rear of lymphocytes during crawling [15] and (2) molecules on the surface are carried on a forward directed path during synapse assembly. Notably, this latter, forward path is smooth and not jumpy [8]—if the roach motel mechanism were in force, instantaneous movements of beads would have both forward and backward trajectories in the course of reorienting into the synapse whereas their observed movement shows steady forward progression [8]. During synapse assembly, it is likely that a directed mechanism must cooperate with a cytoskeletal 'thaw' cycle that permits the movements to occur [8,33].

The concept then that an active actin-based process, independent of cytoskeletal assembly, aids in moving proteins to where they need to be is highly appealing. In the context of actin filaments, myosin motors represent very likely candidates for players involved in actin-dependent molecular movement. The concept that this family of motors is important for surface protein distribution has been around for over 25 years [34,35] but progress with assessing their function has been hampered by the lack of knowledge of which specific motors are present in lymphocytes as well as a paucity of specific reagents for inhibiting motors in general.

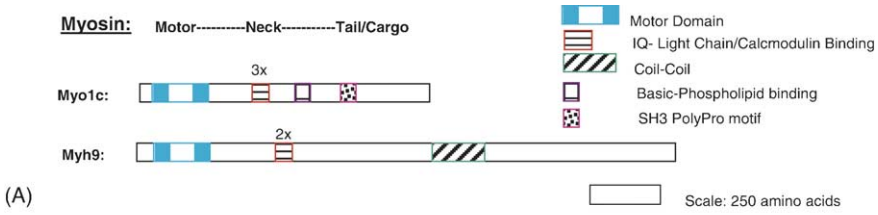
The myosin superfamily comprises 30–40 members in mammalian genomes and is well defined by a three-domain structure, outlined in Fig. 4A. Akin to a train riding on a track, myosin molecules contain a motor domain at their N-terminus which has a region for binding actin as well as an ATPase domain which catalyzes the powerstroke. Just behind the motor sits a prototypical regulatory domain, termed the IQ domain in recognition of the Isoleucine-Glutamine repeats that bind calmodulin or myosin light-chains and thereby influence motor and/or tail functions. Finally, at their C-terminus myosins have a tail domain capable of mediating homo-multimerization with other myosin molecules, as in the case of Class II myosin molecules and/or binding 'cargo' as is thought to occur with the unconventional non-muscle myosin motors.

Assessment of myosin superfamily expression in T cells via gene chip analysis reveals strong candidates for modulating surface events (Fig. 4B). Two classes of motor proteins associated with the plasma membrane are found in T cells, a Myosin II isoform MyH9/NMMHC-IIA and Myosin I isoforms. Most notably, both of these classes of myosins bind directly to membrane lipids in their tail domain [36,37]. This makes them particularly appealing for players that may regulate the flow and segregation of integral membrane proteins, particularly those solvated by lipids that are also selected by the motor protein.

6.1. Class II myosins

T cells express a single class II myosin, MyH9/NMMHC-IIA, a protein whose tail domain contains a predominant coil–coil domain for oligomerization and whose homologs are found associated with the cortical cytoskeleton. The coil–coil nature of the tail allows homodimers of Myosin II family members to form large oligomers, which through concerted action are able to bind adjacent actin filaments and contract one toward the other, thereby enhancing tension in key parts of the cell body. One particular instance in which myosin II plays a role is during cytokinesis where it is necessary for pinching the cleavage furrow to segregate the daughter cells. Blockade of myosin II isoforms in mammalian cells results in multinucleate giant cells as a consequence of the inability to complete cytokinesis [38].

In *Dicytostelium*, retrograde flow in crawling cells is partially controlled by myosin II motors and these are also necessary for efficient motility. Notably, accumulation of concanavalin-A labeled receptors into the rear of migrating *Dictyostelium* similarly requires myosin II. In T cells, we have observed myosin II moving in packets emanating from membrane protrusions at the leading edge and moving back-



(A)

(B)

Motor:	Myosin II: (MyH9/NMMIIA)	Myosin I family
Type	Conventional Filamentous	Unconventional Monomeric
Tail Binding	Other Myosin II molecules Membrane Lipids	Membrane phospholipds, unknown cargo
Interaction with Transmembrane Proteins	Potentially, likely indirect.	Potentially indirect via PolyPro-SH3 interactions (B2) or lipid interactions
T cell Localization	Cortical, enriched at the cell body-uropod junction, in packets following pseduopodial retraction	Cell surface, enriched in the uropod and in developing pseudopods.
Modulation	Light Chains activated by Ca^{2+}. Light Chains phosphorylated in response to Ca^{2+}. Heavy Chains phosphorylated in response to Ca^{2+}	Calmodulin binding in IQ domains. Modulation of motor and/or lipid binding.

Fig. 4. Two classes of myosins are good candidates for influencing membrane lipid and protein dynamics. The top panel outlines the general domain structures for Class I and Class II myosins. The lower panel elaborates the details of each class.

wards toward the uropod [11]. This localization suggests that tensioning and treadmilling mediated by this motor is the key to both overall retrograde flow but also the initial rear biasing of the TCR toward the uropod. However, despite this critical role for myosin II during the crawling phase, it appears to be unimportant in establishing forward directed membrane movement during synapse formation. In fact, TCR signaling results in phosphorylation of this motor in its coil–coil domain, a site associated with controlling multimerization [11,39]. This observation has led to the hypothesis that 'turning off' the tensioning and retrograde flow function of this motor may, in fact, be a prerequisite for T cell 'stopping' in response to antigen engagement. In the absence of such depolymerization, the membrane component flow mediated by the highly processive myosin II motor may prove too overwhelming for a forward-directed motor to overcome.

6.2. Class I myosin isoforms

While class II myosins appear to be good candidates for influencing retrograde flow and the uropodal distributions of transmembrane receptors during amoeboid crawling, a different motor is apparently required for forward membrane movement during synapse formation. T cells express at least one myosin I isoform and these molecules have a number of features which suggest that they may function to move proteins toward the synapse and/or mediate assembly of active complexes into organized clusters. First, they associate with the negatively charged membrane lipids via their C-terminal tail domain. This interaction is positively regulated by intracellular calcium levels, calcium rises being a very early event in T cell signaling. In addition, they are plus-end directed motors, suggesting that they would move membrane-bound components toward the leading edge. Finally, they also contain putative SH3 or SH3-binding domains in their tail giving them a further mechanism for interacting with signaling-regulated protein domains. It remains to be determined if these players counteract the retrograde tensioning function of Class II motors and aid in synapse assembly.

7. Microscopy-based screening approaches to defining cell biological mechanisms underlying transmembrane protein localization

While we have learned much as a result of basic investigations of synapse-assembly dynamics and we have strong candidates in the myosin family for mediating a specific molecular movement issue during synapse assembly, it remains highly likely that entire classes of proteins are present in T cells that function downstream of T cell signaling and that participate in solidifying the cellular conformation of an activated complex. While this prediction cannot be strongly supported with existing data, we will propose an axiom that very few cellular processes proceed in the absence of co-factors to aid in the process. In the case of synapse assembly alone agrin, SNARE proteins and cofilin, all T cell non-specific proteins,

have been shown to function at some level in supporting T cell coupling and signaling [17,40,41]. Furthermore, taking the myosin family as an example, a number of these proteins are likely to be ubiquitously expressed, used in multiple stages of the T cell lifecycle, and therefore unlikely to be 'hits' in differential gene analyses. How many other T cell non-specific, ubiquitously expressed signaling proteins are used to solidify signaling structures?

One highly successful method for identifying proteins involved in a process can be gleaned from elegant phenotypic visual screens carried out in yeast and in mouse embryos. A phenotypic visual screen here is defined as one in which a particular aspect of biology is visually scored, either using a morphological criterion or a visible marker (e.g. a fluorophore). In an ongoing example in higher eukaryotes, Skarnes and co-workers have visually screened promoter-less *lacZ* gene-insertions into embryonic stem cells to find genes that are differentially expressed at key stages of embryonic development. This screen has revolutionized the ability to capture genes in the pathway while identifying hundreds of previously unrecognized players in stem cell differentiation [42]. While such screens can be technically demanding and/or time consuming they nevertheless represent a powerful method for identifying genes involved in particular pathways.

7.1. The immunological synapse as a 'biosensor'

Productive T cell signaling is associated with the recruitment of a multitude of proteins to a signaling 'cap' or 'immunological synapse' [43,44]. Although the accumulations are highly dynamic [6], the flattened interface morphology of the synapse and the accumulation of fusion proteins at this site is easily and reliably scored and has been productively used as an indicator of T cell reactivity [3]. Additionally, at later states of activation, the T cell/APC interface can be observed as organized concentric protein arrays [1,2].

An emerging theme from the study of the immunological synapse is that a vast majority of productive cell–cell signaling goes through this cap. More importantly, the relevant players in the process must be recruited to this site in order to function (most signaling requires that the reactants be in very close proximity and a synapse appears to serve as a hub for biochemistry). We have recently had reason to consider this as our studies of molecular motors in

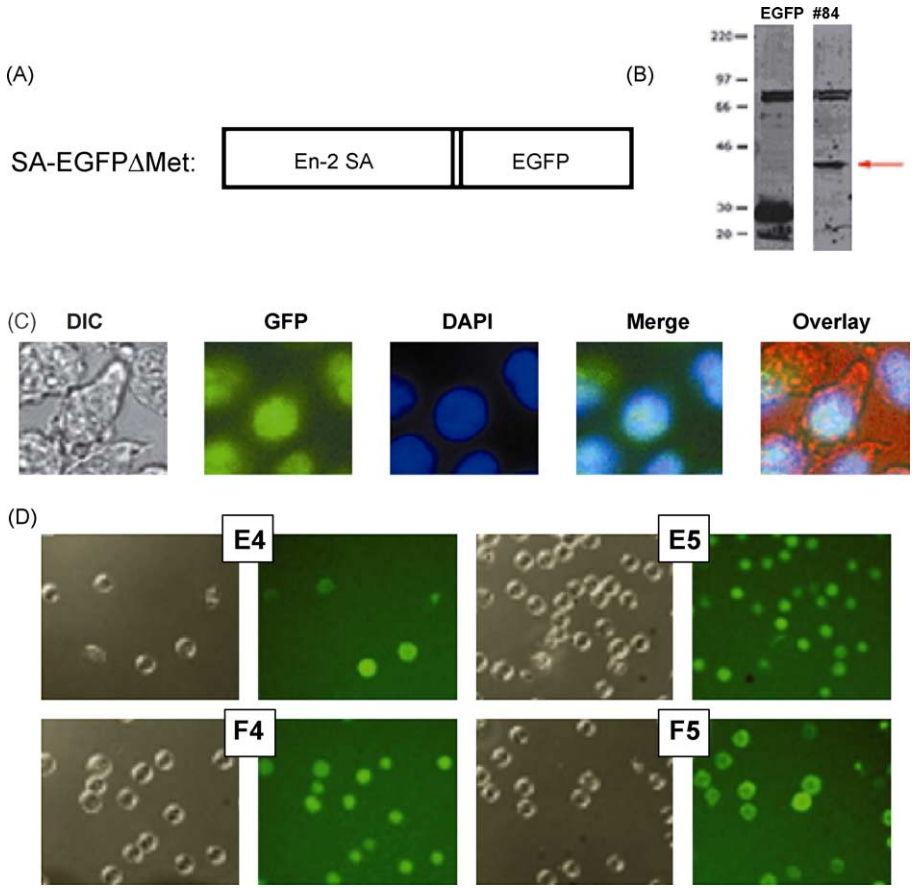


Fig. 5. Imaged-based screening for identification of cell-biology components recruited to the immunological synapse. (A) A construct based on the pGTO,1,2 series of Skarnes et al. [46] in which GFP is substituted for *lacZ*. (B) Production of fusion protein in a clone (#84) assessed by western blotting with anti-GFP. Lysates from cells transfected with pEGFP (clontech) or the construct shown in (A) were lysed and western-blotted using anti-GFP antibodies. The band at 30 kDa in the EGFP lane is wild-type GFP and a new band at ~40 kDa is produced by the fusion. The band at 70 kDa is non-specific. (C) Phenotype of Trap clone #84 shows nuclear localization. pEGFP is typically cytoplasmic. (D) Results from automated plate-screening. DIC and widefield fluorescent images are shown—well numbers from a 96-well dish are given at the top of each image-set.

T cell activation has demonstrated that, of the many myosins expressed in T cells, all are present at relatively fixed levels during development and activation (by gene chip analysis). Only some of these redistribute to the signaling pole and are involved in migration and synapse formation. Since these are all constitutively expressed at the RNA level, the functionally relevant players would not be identified using conventional expression profiling.

7.2. Image-based screening systems

Beyond the purely analytical approaches that live video microscopy has permitted, a novel use of high-frame rate, high sensitivity instruments for phenotypic screening of cell behavior and signaling is becoming apparent. A widefield or high-speed confocal system, equipped with a motorized stage capable of carrying 96- and 384-well plates and controlled by software becomes an ideal instrument to screen large libraries of genes and drugs. Indeed, this is an odd area in science in which more than five companies have developed platforms for automated imaging analysis while largely struggling to find robust biological application. In part this represents biology, which is not always robust enough on a single-cell scale to be used in some of these assays. In part, it represents the sometimes daunting task of assembling large libraries and, most importantly, writing algorithms to handle and pre-screen such large data sets.

In Fig. 5, we outline details of one such ongoing approach that we are undertaking. A base construct that we have extensively engineered using a *lacZ* construct first used by Skarnes and co-workers (Fig. 5A) is used to transfect a T cell clone. Selection for green fluorescence by FACS identifies those cells in which integration results in an in-frame fusion. In Fig. 5B, we show a western blot demonstrating a T cell clone expressing a fusion protein derived by random insertion of this construct into the adenosine kinase gene. Adenosine kinase is a nuclear scavenger and as anticipated, the fusion protein localizes to the nucleus in T cells as shown in Fig. 5C. Finally, Fig. 5D gives a flavor for the output of a number of wells from a 96-well analysis. In this instance, nuclear, cell surface and cytoplasmic phenotypes are visualized.

Given the frequent re-use of mechanisms in biology, it is likely that a number of the key molecules involved in autologous junctions (e.g. adhesive junctions, tight junction complexes, etc.) will play a role in the IS. It has already been established that a number of the extracellular players involved in neurological synapses play conserved functions in the IS. It remains to be determined which other ‘junctional’ mechanisms are also retained for building this specialized T cell signaling array.

8. Concluding remarks

While we are still a ways off the mark with respect to understanding the many players that underlie the cell biology of T cell responses, the synapse represents an ideal place to start. It is clearly a point at which great change occurs for the T cell. It converts from a nomadic, motile existence to a part of a cell–cell interaction. It completely changes morphology. Membrane distributions change and the cytoplasm is reoriented. Having previously integrated chemotactic signals at its leading edge, it now converts to both receiving and sending its own signals, particularly those of secreted cytokines and exposed cell surface receptors [45]. While the smallness of the T cell has never attracted cell biologists, it is clear that this is a system in need of better characterization and understanding at this level.

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