

Cell surface organization by the membrane skeleton

Akihiro Kusumi* and Yasushi Sako†

Single-particle tracking and laser tweezers have facilitated the observation of the mechanics of molecular interactions in the plasma membrane of living cells at the level of single (or a few) molecules at nanometer/piconewton precision. These techniques have recently revealed that the membrane skeleton provides both confining and binding effects on the movement of membrane proteins, and that it can play a pivotal role in the molecular organization of the plasma membrane, especially in the formation of special membrane domains.

Addresses

Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba 3-8-1, Meguro-ku, Tokyo 153, Japan
*e-mail: akusumi@kusumib.c.u-tokyo.ac.jp
†e-mail: sako@kusumib.c.u-tokyo.ac.jp

Current Opinion in Cell Biology 1996, 8:566–574

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Abbreviations

BFP	barrier-free path length
FRAP	fluorescence recovery after photobleaching
GPI	glycosylphosphatidylinositol
MDCK	Madin–Darby canine kidney
NRK	normal rat kidney
pN	piconewton
SPT	single-particle tracking
TR	transferrin receptor

Introduction

Cells build various structures and arrays of proteins and lipids, within and around the plasma membrane, that are essential for the proper functioning of both these molecules and the plasma membrane. These supramolecular complexes include: multimers of receptor molecules or receptor and effector molecules that are thought to be the first trigger for the cellular reactions that follow ligand binding [1,2,3^{••},4^{••}]; specialized membrane domains, such as synapses, clathrin-coated lattices and pits, caveolae, and cell–cell and cell–substrate adhesion structures, in which membrane domains specific proteins and lipids are assembled to carry out specific functions [5[•],6[•],7]; and the polarized distribution of various proteins in epithelial and neuronal cells [8]. The constituent molecules of such supramolecular structures are recruited and assembled from the plasma membrane and the cytoplasm, and by intracellular vesicular transport. In this review, we are mostly concerned about the recruitment of membrane proteins to specialized domains within the plasma membrane.

In the recruitment, multimerization, and assembly of specific membrane proteins and lipids, one of the critical processes is the regulation of the movement of these

molecules. Proteins are not free-floating in a sea of excess lipids; in other words, the cells have various means by which they control the mobility and specific assembly of membrane proteins in specialized domains. In this article, we will present a brief review of recent findings relevant to the mechanisms that control the movement and assembly of integral membrane proteins.

Our understanding of the cellular mechanisms that control the movement and assembly of membrane proteins in the plasma membrane is currently undergoing rapid evolution. This is mainly due to the application of newly developed light microscopic techniques, such as single-particle tracking and laser tweezers, to studies of the molecular dynamics of the plasma membrane. Reviews of these methods have been published [9,10,11^{••}].

It is becoming clear that the non-homogeneous distribution and assembly of membrane proteins in the plasma membrane are, in part, regulated through the membrane-associated portion of the cytoskeleton, that is, the membrane skeleton. As a consequence, more attention is being directed toward understanding membrane–cytoskeleton interactions and their roles in regulating the architecture of the plasma membrane [12–16]. Of particular interest is the involvement of membrane-skeletal elements in mediating or inhibiting movements of cell surface receptors, and their participation in the formation of specialized domains and in signal transduction in the plasma membrane. Recent relevant reviews have covered aspects of cytoskeleton–membrane interactions [17], cytoskeletal control of lateral diffusion of membrane proteins [11^{••},18^{••}], and localization of membrane proteins during polarization development [8].

Here, we will review the roles that the cytoskeleton/membrane skeleton may play as an organizer of molecules in the plasma membrane. In addition to the cytoskeleton/membrane skeleton, the multimerization and aggregation of membrane proteins [4^{••},19], the partitioning or binding of proteins to specific lipid domains [20^{••}], vesicular transport and fusion at specific sites in the plasma membrane, and signal-transduction systems including kinases and G proteins [6[•],7] are important for cell surface organization, but, due to space limitations, we will touch upon these subjects only briefly as we discuss a possible mechanism for the formation of membrane domains. We will primarily concentrate on the cell surface organization by the cytoskeleton/membrane-skeleton network, and we will develop a model in which regulation of the movement of membrane proteins by the membrane skeleton leads to the formation of specialized membrane domains.

Single-particle tracking has revealed various modes of protein motion in the plasma membrane

In single-particle tracking (SPT), the movements of single nanometer-size colloidal gold or fluorescent particles that are bound to a single (or a small number of) molecule(s) are followed by optical microscopy with the possibility of nanometer-level precision [11**,21–26]. Qian *et al.* [27] and Saxton [28,29,30**,31] have presented a theoretical basis for analyzing the trajectories obtained by SPT. Kusumi *et al.* [32] have described a practical approach for the analysis of single-particle trajectories in order to distinguish between true deviations from Brownian motion and the statistical fluctuations inherent to random thermal motion. Using this approach, Kusumi *et al.* [32] and Tomishige [33] found that the means of movement of many membrane proteins can be classified into the five types of motion that are believed to occur in the plasma membrane: first, stationary mode; second, simple Brownian diffusion mode; third, directed diffusion mode; fourth, confined (or corralled) diffusion mode (a particle undergoing free diffusion is confined within a limited area); and fifth, diffusion in a harmonic-like potential (a protein bound to an elastic cytoskeleton will diffuse in this manner) [32,33]. A single protein molecule can change its motional modes in time [32,34,35**]. Finding the portions of confined diffusion is important because they may be related to membrane domains. Simson *et al.* [35**] have developed a useful method to detect the portion of a single-particle trajectory in which the particles are undergoing confined diffusion.

The nanometer-level precision of SPT is particularly useful for studying the molecular mechanisms that are working at the submicron scale, such as in the spectrin network of erythrocytes. Measurements of both lateral and rotational diffusion were previously needed to obtain information at the nanometer or molecular level [36–38], but these measurements can be omitted if SPT is employed.

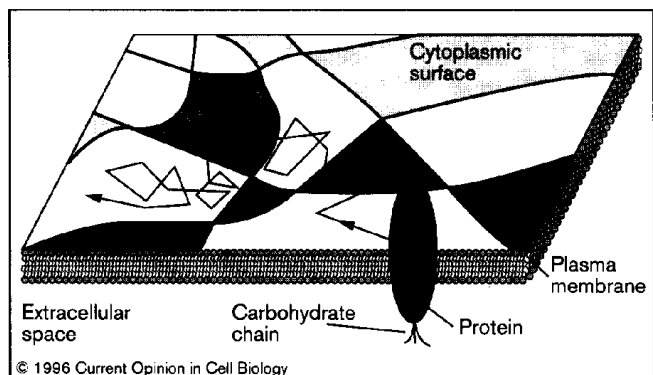
Nevertheless, comparison of SPT data with the results of fluorescence recovery after photobleaching (FRAP) is still useful. In addition to examining the possible artefacts associated with gold labeling, the cellular structures that regulate the mobility of membrane proteins can be examined on a scale from a nanometer to over a micrometer by applying both techniques to the same system [32]; A Tsuji, Y Sako, M Tomishige, A Kusumi, unpublished data).

Single-particle tracking has shown that the plasma membrane is compartmentalized with regard to lateral diffusion of transmembrane proteins and that many membrane proteins undergo intercompartmental hop diffusion

SPT studies showed that the plasma membranes of a variety of mammalian cells, such as normal rat kidney (NRK)

fibroblastic cells, Madin–Darby canine kidney (MDCK) epithelial cells, and mouse keratinocytes, are compartmentalized into many small domains of 0.1–1 μm^2 (with diameters of 0.4–1.2 μm) with regard to the lateral diffusion of many membrane proteins (see Fig. 1), including transferrin receptors (TRs), α_2 -macroglobulin receptors, epidermal growth factor receptors, E-cadherin, and Na^+ , K^+ -ATPase ([32,39]; A Tsuji, Y Sako, M Tomishige, A Kusumi, unpublished data). The TR and the α_2 -macroglobulin-receptor molecules in the plasma membrane of NRK cells are confined within these compartments for 25 seconds on average and then hop to an adjacent compartment. Within a domain, these receptor molecules undergo rapid lateral diffusion that is indicative of free diffusion (the microscopic diffusion coefficient is $\sim 10^{-9} \text{cm}^2/\text{s}$); the rate of long-range diffusion of these receptors is less by a factor of 30. The macroscopic diffusion rate is determined by the size of the compartment and the frequency of intercompartmental hops, which is irrelevant to the microscopic diffusion rate. Partial destruction of microfilaments or microtubules dramatically changes the motional modes of these receptors [39].

Figure 1



A model showing the compartmentalized structure of the plasma membrane and intercompartmental hop diffusion of membrane proteins. The plasma membrane is compartmentalized into many domains of 0.1–1 μm^2 for diffusion of transmembrane proteins; these domains are represented here by a series of variously shaded shapes. The receptor molecules undergo almost free diffusion within a compartment (slowed only by the presence of other membrane proteins) to which they are confined for an average of 25 seconds. Movement both between and within compartments is represented by a thin black arrowed line. The receptor molecules move from one compartment to one of the adjacent domains at a frequency of 0.04 s^{-1} (2.4 intercompartmental hops/min), on average, and the long-range diffusion of receptors occurs as the result of successive intercompartmental movements. Therefore, the macroscopic diffusion rate (D) is determined from the size of the compartment and the frequency of jumps between compartments, and gives the macroscopic D of $2.4 \times 10^{-11} \text{cm}^2/\text{s}$.

Compartmentalization of the plasma membranes was also suggested in PTK2 cells by SPT [40], and in human skin fibroblasts (CCD cells) and K78-2 hepatoma cells by

FRAP, in which diffusion was observed as a function of the size of the bleaching and observation spot [41,42].

Use of laser tweezers together with single-particle tracking has revealed the presence of elastic intercompartmental barriers

Gold particles attached to membrane constituent molecules work not only as probes but also as a handle with which to move these molecules along the plasma membrane by the use of a single-beam gradient force optical trap, also referred to as laser tweezers or optical tweezers [9,10,43,44]. By using the laser trap to apply restraining forces to particles, we are able to capture, move, and release gold-particle-protein complexes in the membrane at will. For example, Kucik *et al.* [45] and Schmidt *et al.* [46] used laser tweezers to move particle-glycoprotein complexes to various locations on the cell surface and to restrain bead movement at specific locations in the cell. If an infrared laser is used, the intensity levels required to control particle positions cause negligible damage in living cells. Living bacteria and yeast cells have been held in a laser trap for five hours without apparent damage, and were even observed to reproduce while trapped [47].

Edidin *et al.* [48] moved transmembrane MHC class I (H-2D^b) and glycosylphosphatidylinositol (GPI)-anchored MHC class I (Qa2) molecules in the plasma membrane. The protein-gold-particle complexes were dragged by laser tweezers until they escaped from the trap when they encountered barriers. Thus, the barrier-free path lengths (BFPs) could be obtained. They were 3.5 and 8.5 μm for the transmembrane and GPI-anchored species, respectively, at 34°C. The BFP of a mutant H-2D^b molecule, which possesses only four amino acid residues in the cytoplasmic domain (vs. 31 residues in the wild type), was about twofold greater than that of the wild type [49]. These results suggest that the cytoplasmic domain of the transmembrane protein is involved in the regulation of lateral movements of these proteins.

At lower trapping forces, compartments of comparable sizes to those observed by SPT could be detected [50••]. At a maximal dragging force of 0.25 to 0.8 piconewtons (pNs), TR molecules that are undergoing confined diffusion with a high microscopic diffusion coefficient (D_{micro}) of $10^{-9} \text{cm}^2/\text{s}$ (this applies to 90% of TRs in NRK cells) could be dragged past the intercompartmental boundaries in their path. At lower dragging forces of between 0.05 pN and 0.1 pN, TRs tended to escape from the laser trap at the boundaries, because the force from the boundaries that acts on TRs is greater than these dragging forces. Such escapes occurred in both the forward and backward directions of dragging; this cannot be explained by the tethering of TRs to the cytoskeleton. The BFP for each molecule was, on average, half of the confinement size estimated in SPT observation which further indicates that TRs escaped at the compartment boundaries (as

the average start point of dragging is the center of the compartment, the dragged distance will be half the compartment size if TRs always escape at the boundaries).

When TR molecules escape from the laser trap, they almost always spring back rapidly in either their forward or backward trips. The rebound quickly ceases and the particle begins to exhibit Brownian diffusion in less than 0.2 seconds. These results suggest that the compartment boundaries are elastic in nature. The effective elastic constants of the boundaries are in the range of 1–10 pN/ μm .

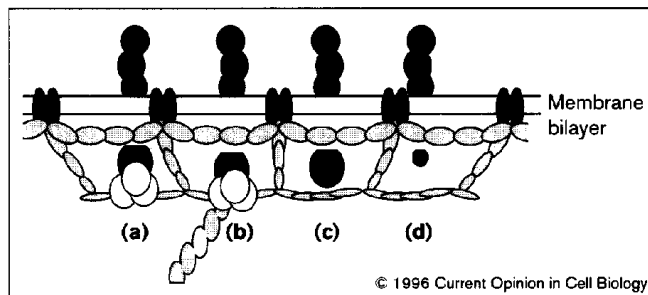
Membrane-skeleton fence model

As variations in the size of the attached gold particle (the particles are either 40 or 210 nm in diameter and are on the extracellular surface of the plasma membrane) hardly affect the protein's diffusion rate and behavior in the dragging experiments, and as treatment with chemicals that destroy either actin filaments or microtubules affects the movements of TRs, the boundaries are likely to be present in the cytoplasmic domain [39,50••]. The rebound of the particle-TR complexes when they escape from the laser tweezers at the compartment boundaries suggests that the boundaries are elastic structures. These results are consistent with the proposal that the compartment boundaries consist of a membrane-associated portion of the cytoskeleton. This proposal is the membrane-skeleton fence model (see Fig. 2).

In this model, the membrane skeleton provides a barrier to free diffusion of membrane proteins due to steric hindrance (the space between the membrane and the cytoskeleton is too small to allow the cytoplasmic portion of the membrane protein to pass), thus compartmentalizing the membrane into many small domains (see Fig. 2) of 0.1–1 μm^2 [11••,18••,32,39,50••,51,52•]. The membrane proteins can escape from one domain and move to adjacent compartments as a result of the dynamic properties of the membrane skeleton: the distance between the membrane and the skeleton may fluctuate over time (or the membrane skeleton may dissociate from the membrane), or the membrane-skeleton network may form and break continuously as a result of dissociation-association equilibria, thus giving the membrane proteins an opportunity to pass through the mesh barrier. Furthermore, the membrane protein molecules that have sufficient kinetic energy will be able to cross the compartment boundaries.

Confined lateral diffusion and intercompartmental hop diffusion of membrane proteins have been observed for a variety of membrane proteins in all cells studied thus far. We propose that compartmentalization of the plasma membrane by a membrane-skeleton/cytoskeleton meshwork (membrane-skeleton fence structure) is a basic feature of the plasma membrane. For individual protein species, more specific mechanisms such as direct binding to the cytoskeleton may be at work. However, what should be emphasized here is that the generalized fence effect of

Figure 2



Membrane-skeleton fence model. In this figure, the plasma membrane is viewed from inside a cell. The bilayer portion of the membrane, the transmembrane proteins (in black) and those connecting the membrane skeleton to the membrane (these proteins are shown in dark gray shading, and the membrane skeleton in light gray shading), the cytoplasmic proteins (open circles in [a] and [b]) bound to membrane proteins, and the binding of this complex to the cytoskeleton (b) are shown. The membrane skeleton is in close proximity to the cytoplasmic surface of the plasma membrane. When the membrane protein diffuses in the membrane, its cytoplasmic domain (or its complex with a cytoplasmic protein) will collide with the membrane skeleton and will not be readily able to move to an adjacent compartment (see [a-c]). If the cytoplasmic domain is smaller (d), the protein can move to an adjacent compartment more readily. Binding of membrane proteins to the membrane skeleton (see [b], but note that direct binding of transmembrane proteins to the membrane skeleton also occurs) has been found for a variety of proteins. Binding can be detected by using laser tweezers to drag the membrane protein. In many cases, the membrane skeleton itself is undergoing macroscopic diffusion in addition to oscillative motion without any real displacement. Some membrane skeletons show directed, active transport type movements, and the transmembrane proteins that are bound to such skeletons also undergo similar directed-type movements.

the membrane skeleton is superimposed on the specific effect that applies to individual protein species. In the case of E-cadherin, some molecules that are bound to the flexible cytoskeleton, possibly thin actin filaments, 'feel' the presence of the membrane-skeleton fence as they move about with the attached cytoskeleton (Y Sako, A Kusumi, unpublished data).

Binding and transport of membrane proteins by the membrane skeleton

Almost all membrane proteins investigated so far have been found to bind to the membrane skeleton, including the receptors for transferrin, epidermal growth factor and $\alpha 2$ -macroglobulin, E-cadherin, T-cadherin, and the band 3 anion channel in erythrocytes (Fig. 2b shows the binding of a membrane protein to the membrane skeleton) [32,33,39,53,54]. (The strength of the interaction between the cytoskeleton and the membrane bilayer in neuronal cells has been estimated to be 2–8 pN [55].) These bound proteins undergo various types of motion. Some show no motion, while others show oscillative movements without real displacement in space. Some proteins show long-range translational diffusion (lateral diffusion) while they are apparently bound to the membrane skeleton (it is known that these particles are bound to the membrane skeleton

because they cannot be dragged more than 100 nm by laser tweezers). Some show directed transport type movements, probably as a result of the active movement of the cytoskeleton to which they are bound.

The mechanism by which cells control these processes and exert the fence effect of the membrane skeleton has yet to be elucidated. The amount of E-cadherin bound to the membrane skeleton decreases after a calcium switch [32]. The size of the compartment as 'felt' by the Na^+, K^+ -ATPase in the dorsal/apical membrane decreases by a factor of 2 after the calcium switch in MDCK cells (A Tsuji, Y Sako, M Tomishige, A Kusumi, unpublished data). Specific binding to a particular membrane skeleton/cytoskeleton may also be controlled by phosphorylation. It is likely that cells are using the fence effect and active transport by the membrane skeleton/cytoskeleton to assemble specific membrane proteins into specialized domains. However, exactly how cells do this is not known, and is one of the most important issues in membrane biology.

Various force potentials that act on membrane proteins have been revealed by studying their translational diffusion

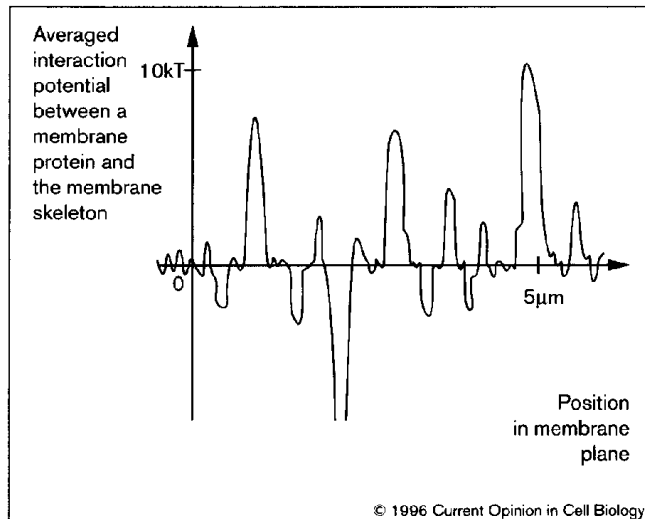
Various mechanisms for the regulation of the long-range translational movements (those of more than $1 \mu\text{m}$) of membrane proteins have been proposed (for reviews, see [15,18^{**},56]). These include: first, a percolation mechanism based on the presence of the static and slowly moving components of membrane proteins and the domains of solid lipids and those rich in proteins, both of which greatly inhibit free motion of membrane proteins as they occupy 10–30% of the membrane area [11^{**},42,57–60]; second, the interaction of the extracellular domains of the membrane proteins with other cell surface components [61,62]; third, a crowding effect of the extracellular domains of membrane proteins [57], possibly due to the large exclusion volume of the carbohydrate chains [63]; fourth, a corralling effect of the membrane skeleton [11^{**},18^{**},36,37,39,50^{**},51,59,64]; fifth, tethering to the membrane skeleton [50^{**}]; and finally, dynamic association with other membrane proteins and the membrane skeleton (a fragile time transport process, as named by Webb [65]).

Oligomerization of membrane proteins is also likely to be involved in the regulation of long-range diffusion. Oligomerization *per se* does not decrease the rate of translational diffusion (D_{micro}) in two-dimensional space. However, as oligomerization is very likely both to decrease the rate of hops from one compartment to an adjacent compartment and to increase the probability of binding of a protein to the membrane skeleton, the macroscopic diffusion rate is likely to be decreased as a result of the oligomerization of membrane proteins.

Membrane proteins diffuse in the plasma membrane and 'sample' the various force potentials (see Fig. 3) and microscopic viscosities that are a result of the mechanisms

described above. These potentials and viscosities and their spatial variations can be found by studying the diffusion of proteins in the plasma membrane.

Figure 3



A schematic drawing showing a snapshot of potential wells (attractive force potentials, having negative values) and barriers (repulsive force potentials, having positive values) that may act on a protein in the plasma membrane. The noise level, due to thermal fluctuations, of about $kT=4\text{pN/nm}$ on average is assumed to be averaged in this figure.

In this review, what we wish to emphasize is that among the repulsive and attractive potential fields (barriers and wells, respectively) for the diffusion of membrane proteins, those that are a result of the interaction with the membrane skeleton are far greater than those that are not. Furthermore, cells can readily regulate the barriers and wells formed by the membrane skeleton/cytoskeleton by varying their structures and properties; this is important in order that the cells can respond to extracellular signals and adapt to environmental changes.

Control mechanisms for the formation of supramolecular arrays and assemblies in and around the plasma membrane

We envisage three basic processes for the assembly of membrane proteins through movements in the plasma membrane. These are basic concepts and are not mutually exclusive. First, diffusion of the membrane protein and entrapment of the protein at specific sites in the membrane can occur, possibly due to the preassembly of cytoplasmic proteins on the cytoplasmic surface of the membrane. Cooperative assembly of intramembrane proteins and peripheral proteins is a possibility. Second, cells take advantage of thermal diffusion to drive the movements of membrane proteins, but regulate the direction of the movements by varying the structure of the membrane

skeleton using free energy released by hydrolysis of ATP. In this working hypothesis, we postulate that the free energy generated by ATP hydrolysis is used not to drive the movements but rather to regulate them. The basic idea for this hypothesis is that, rather than allowing simple self-assembly of molecules, cells actively regulate thermal movements to construct supramolecular complexes. Third, gross, active movements of the membrane-skeleton network may occur to move the membrane proteins trapped in the compartments (like many fish in a fishnet) or those bound to the skeletal network. For example, we envisage that oligomers and aggregates are bound to the membrane skeleton and are carried by the skeleton as a single cargo. The key idea is that cells would not move membrane proteins one by one because it is both energetically and temporally too inefficient.

We believe that the cytoskeleton/membrane skeleton works as an organizer of molecules in the plasma membrane. In addition to regulating the movements of membrane proteins, the membrane skeleton/cytoskeleton may regulate other undercoat structures of the plasma membrane such as caveolae and clathrin-coated structures. The association of F-actin with these structures has been reported [54,66].

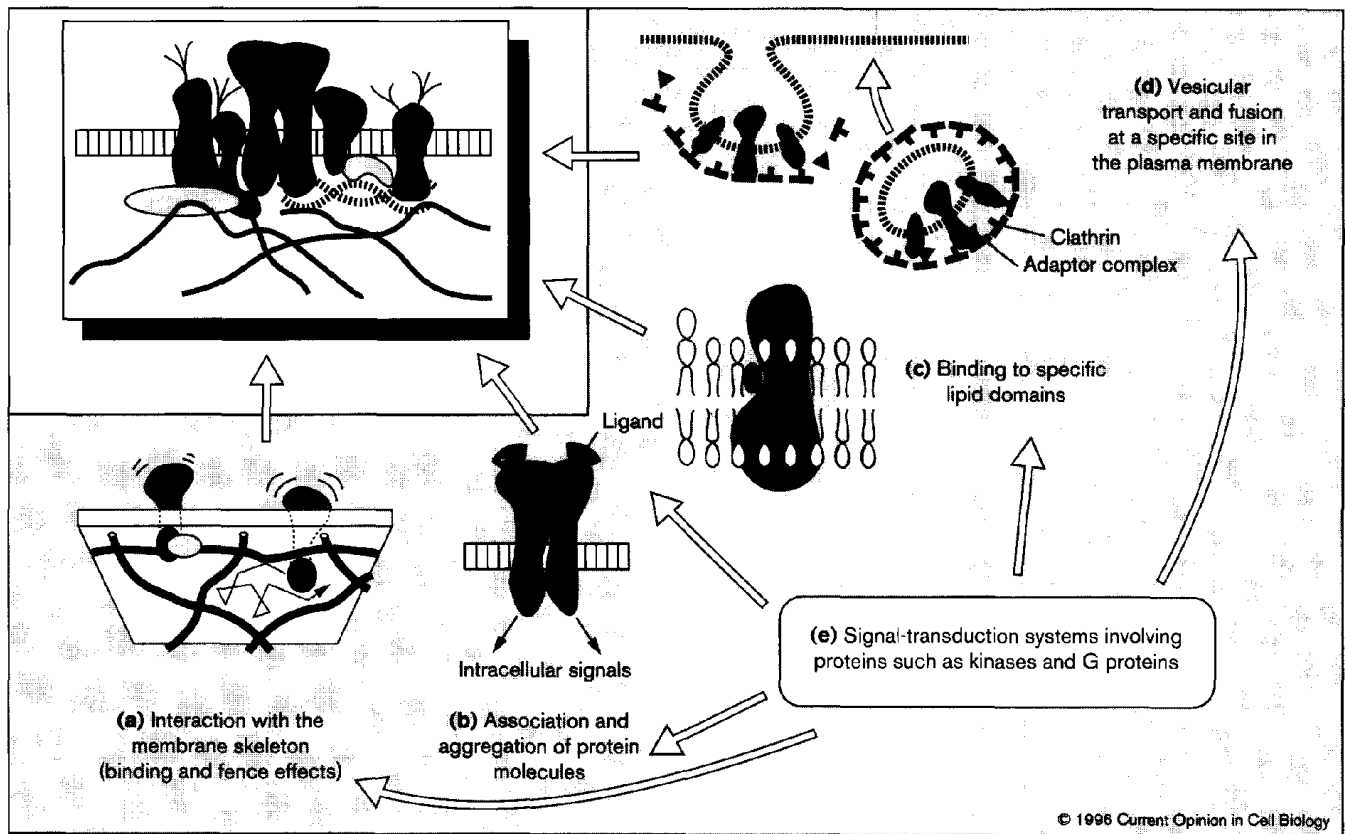
Other processes would also be involved in the assembly of membrane proteins (Fig. 4). Multimerization and aggregation of membrane proteins can greatly increase the interaction of the membrane proteins with the membrane skeleton, as discussed above. Partitioning or binding of proteins to specific lipid domains may be a critical step, particularly in the case of caveolae formation [20**,67,68**,69**]. Vesicular transport and fusion at specific sites in the plasma membrane may be important for the formation of caveolae [20**] and cell adhesion structures [70], and for the polarized distribution of proteins in epithelial cells. For the initiation and regulation of the formation of various supramolecular structures, signal-transduction systems including kinases and G proteins must play key roles.

Presently, in studies of membrane domains, the above processes are usually investigated separately. The central aim of future studies is to integrate our knowledge of these processes into a cohesive understanding. Progress in future research on the formation of supramolecular structures in the plasma membrane will greatly depend on concerted action of biophysicists and cell biologists.

New technologies are opening up possibilities for scientists to study molecular mechanics in cells by directly handling single molecules in living cells

SPT and laser tweezers have opened the way to study the processes involved in the organization of membrane molecules at the level of single molecules. Whereas cells use 'molecular hands' (molecular interactions) to organize

Figure 4



Elementary processes for the formation of supramolecular arrays and assemblies. **(a)** Interactions with the membrane skeleton (binding and fence effects) are the major processes emphasized in this review. Gray strands represent the membrane skeleton. **(b)** Multimerization and aggregation of membrane proteins and **(c)** binding of proteins to specific lipid domains may be critical steps. **(d)** Vesicular transport and fusion at specific sites in the plasma membrane may play important roles. **(e)** For the initiation and regulation of the formation of supramolecular structures and the regulation of the elementary processes shown in (a–d), intracellular signaling systems including kinases and G proteins must play key roles. These processes work in concert to generate supramolecular structures in the plasma membrane (top left-hand corner).

molecules with nanometer precision by forces on the piconewton level, we can now use 'optical hands' to manipulate single molecules with similar spatial precision by using forces of similar levels to interrupt or imitate the molecular hands of cells. Such studies will help to elucidate the mechanisms and principles guiding the formation of supramolecular complexes in the plasma membrane [71••]. Near-field optical microscopy [72••,73••], force microscopy with an ultrasensitive transducer made of a cell-size membrane capsule pressurized by micropipette suction [74••], and the scanning-force microscope which is based on an optical trap [75,76] will also contribute to this line of study.

Conclusions

The membrane skeleton plays a pivotal role in the molecular organization of the plasma membrane. Its fence and binding effects on the movement and assembly of membrane proteins are key mechanisms for cell surface organization. These effects were revealed by new methods, such as SPT and laser tweezers, that can deal with

single (or a few) molecules with nanometer/piconewton precision in living cells. However, further studies are required to elucidate the mechanism of the formation of supramolecular structures and arrays in and around the plasma membrane. First, more information on the mechanism by which cells regulate the membrane skeleton is needed. Second, better understanding of other supramolecular assembly mechanisms, such as oligomerization of membrane proteins, partitioning of proteins to specific lipid domains, directed vesicular transport, and signal-transduction systems, will be necessary. Third, the relative contributions of, and interplay between, these processes should be investigated for each type of supramolecular complex as they will vary for different structures. These studies would lead to an understanding of the mechanisms that regulate the long-range molecular organization of the plasma membrane, a problem which continues to be very challenging.

Acknowledgements

We thank Takahiro Fujiwara for help with preparing Figures 3 and 4, and Paul Wiseman for his critical reading of the manuscript.

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