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SIGNALING NETWORKS IN LIVING CELLS

Michael A. White and Richard G.W. Anderson
Department of Cell Biology, University of Texas, Southwestern Medical Center, Dallas, Texas 75390-9039; email: Richard.Anderson@utsouthwestern.edu

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■ **Abstract** Recent advances in cell signaling research suggest that multiple sets of signal transducing molecules are preorganized and sequestered in distinct compartments within the cell. These compartments are assembled and maintained by specific cellular machinery. The molecular ecology within a compartment creates an environment that favors the efficient and accurate integration of signaling information arriving from humoral, mechanical, and nutritional sources. The functional organization of these compartments suggests they are the location of signaling networks that naturally organize into hierarchical interconnected sets of molecules through their participation in different classes of interacting units. An important goal is to determine the contribution of the compartment to the function of these networks in living cells.

INTRODUCTION

Enormous progress has been made during the past decade identifying sets of molecular interactions that transmit information between different parts of the cell. The increasing number of databases containing lists of interacting biomolecules has sparked the development of the burgeoning field of network biology and with it the realization that, to a first approximation, biological networks can be described mathematically by scale-free power functions (1). These functions predict the hierarchical interconnectedness of molecules through their participation in different classes of interacting units such as nodes, hubs, modules, and motifs. Power functions mathematically describe the organization of thermodynamically far from equilibrium systems like living organisms. The network structure derived from this type of abstract analysis is very useful for understanding the patterns created by interconnecting the molecular constituents of different signaling pathways. Understanding exactly how these molecular interactions become determinants of cell structure and function, however, remains a significant challenge. The molecular interactions underlying biological networks take place in living cells, and network analysis inherently is unable to consider the contribution of different intracellular environments to signal transduction. Therefore, an important next step is to develop a high resolution map of signaling networks in living cells and the location

of interacting signaling units (i.e., hubs, motifs, modules) relative to cell structures like the plasma membrane, mitochondria, the nucleus, etc.

Cell biologists use many techniques to map the distribution of molecules in cells. Cell fractionation as well as light and EM immunocytochemistry are the principal methods that have been used to demonstrate that cell signaling molecules tend to be concentrated in different cellular compartments. The compartmentalization of interacting sets of signaling molecules has several implications for understanding signaling networks in situ. First, these compartments often can be isolated in a way that preserves the functionality of the resident signaling units. They contain dynamic information about the behavior of molecules that make up specific signaling networks, and embedded in the pattern of molecular interactions are the codes that govern cell behavior. Compartments also contain the molecular signature of unknown signaling pathways that cannot be detected using ex vivo techniques. For example, current estimates indicate that the human genome contains vastly more signaling molecules than have been classified and assigned to pathways. Determining the compartment where these molecules reside is a valuable first step in identifying, mapping, and characterizing their function. Another important consideration is that similar sets of signaling units can be found in different compartments, although the same class of compartments at other times contains different sets of signaling units. Nothing is known about the rules that control the compartmentalization of signaling units, nor how the spatial distribution of these units and the environment created by the host compartment influences signal transduction. Deciphering the rules of compartmentalization can only be achieved by studying the function of signaling units when they are in different host compartments. Each type of compartment is spatially restricted, so compartmentalization also contributes to the spatial organization of signal transduction in the cell. A final consideration is that the mechanism of action of a signaling molecule in a compartment cannot be predicted simply by knowing all its interacting partners. Compartmentalized sets of signaling molecules display emergent behavior that can only be understood by studying the entire ensemble of molecules interacting in their native environment. The fidelity of signal transduction depends as much on the molecular ecology of the compartment as it does on the interaction between individual signaling molecules.

ORGANIZATION OF A SIGNALING UNIT

Efforts to decipher the molecular nature of cell autonomous regulatory programs often, by necessity, progress by collecting the protein components that appear to play obligatory roles in the regulatory process and by determining the biochemical relationships among them. This approach has very successfully identified linear relationships between the interacting components of multiple signaling pathways. Iteration of this process often reveals, however, that branch-points exist in these pathways. These branch-points connect to other independently established

pathways, creating a situation in which diverse inputs (S1, S2, S3–12, Figure 1*A*, see color insert) can transmit through a limited number of core transducers (E1, E2, E3, Figure 1*A*) to multiple outputs (R1, R2, R3, Figure 1*A*). The apparent complexity of this organization raises questions about how these signaling units are able to establish high fidelity coupling between diverse stimuli and discrete physiological effects in the living cell. Moreover, the identity of interacting signaling units in a network does not reveal how these units are spatially and temporally organized in the cell. The ERK1/2 MAP kinase cascade is a well-studied signaling pathway that illustrates the dilemma.

The ERK1/2 protein kinases are a core signaling element implicated in the regulation of assorted biological processes, ranging from cellular proliferation and tumorigenesis to differentiation and cell specialization (2, 3). ERK1/2 apparently function as the terminal kinase in a three-kinase cascade that includes the Mek MAP kinase kinase (MAP2K) and the Raf MAP kinase kinase kinase (MAP3K). This linked set of protein kinases is a signal propagation cassette (E1, E2, E3, Figure 1*A*) typical of many signaling kinase families. ERK1/2 activation can be stimulated by growth factor receptors, heterotrimeric G-protein coupled receptors, and integrins. Many ERK1/2 effector proteins (R1, R2, R3, Figure 1*A*) have been identified, including nuclear transcription factors like c-Fos and Elk-1 (4, 5), cytoplasmic protein kinases like p90RSK (6, 7) and myosin light chain kinase (8), and lipases like phospholipase A_2 (9). Moreover, recent proteomic studies identified 20 new targets for this kinase that are involved in such diverse activities as nuclear import, nucleotide excision repair, membrane traffic, and cytoskeleton assembly (10). The pleiotropic consequences of ERK1/2 activation imply that activated ERK1/2 is directly connected to many different targets.

The ERK1/2 protein kinase cascade is functionally coupled to stimuli in part by Ras family small GTPases (3). Ras proteins are tethered to membranes rich in sensory receptors through carboxy-terminal lipidation (11). The bulk of the kinase elements in the ERK1/2 kinase cascade, by contrast, are in the cytosol of unstimulated cells. In response to stimulus, Ras proteins are activated through GDP/GTP exchange as a consequence of receptor-driven association with guanyl nucleotide exchange factors (GEFs). Ras-GTP adopts a conformation that favors the direct interaction with downstream effector proteins, including Raf kinases (11). Recent structural and biochemical analysis of Ras GEFs (12), together with single-molecule imaging of activated Ras (13), suggests that these enzymes function processively by generating interactive surfaces in the activated state; these surfaces form signaling platforms for combinatorial sets of protein-protein interactions. Normally, activation of Ras recruits Raf-1 to the plasma membrane, but Raf-1 artificially targeted to membranes causes activation of ERK1/2 independently of Ras. Therefore, the current paradigm for Raf-Mek-Erk pathway activation suggests that Ras-GTP acts as "molecular flypaper" that snares Raf kinases at the plasma membrane where they are subsequently activated by other membrane-associated components that have not yet been defined [reviewed in Kolch (14)]. Raf in turn mediates activation of MEK and ERK through a linear cascade of kinase/substrate

interactions (Figure 1*A*). Although this model partially explains how various signals are linked to ERK1/2 activation, it does not clarify how activated ERK1/2 is tuned to the diverse signaling targets it controls.

A growing number of observations suggest that scaffolding proteins (M1, M2, M3, Figure 1*B*) can selectively couple ERK1/2 activation to distinct regulatory programs. Genetic screens for modifiers of the Ras-Raf-Mek-ERK cascade, together with protein/protein interaction studies, have identified proteins like KSR, CNK, MP-1, and Sur-8 that possess no obvious intrinsic enzymatic activities but physically interact with multiple core components of the ERK1/2 cascade (15). These scaffolds appear to be obligate components of ERK1/2 signaling modules (16–18), are required for ERK1/2 activation in cells (19–21), and contribute to the functional coupling of the ERK1/2 cascade to selective stimuli (20, 22). For example, CNK can interact directly with Raf kinases (17), is required for Raf activation in response to insulin in insect cells (21), and functions at least in part to partition Raf into membrane compartments (21). In mammalian cells, the CNK family member CNK2 may help neuronal precursor cells distinguish between neurotrophic and proliferative signals. For example, CNK2 is required for activation of ERK1/2 by TrkA receptor signaling but not EGF receptor signaling (20). The ERK1/2 scaffold Sur-8, by contrast, appears to be more important for EGF receptor signaling (22). These observations suggest that scaffold proteins mediate the assembly of signaling modules that are selectively coupled to discrete receptor inputs (Figure 1*B*).

An additional mechanism for establishing specific input-output connections for these signaling modules may be to spatially segregate each module into a different cell compartment. Thus, the higher-ordered molecular organization generated by scaffold proteins may also function to target the kinases (E1, E2, E3) to a specific location in the cell (Figure 1*C*). This hypothesis would require address information on the scaffolding protein, or on some component of the module, that would target it to a compartment, thereby creating spatially restricted signaling activity. There is considerable evidence that the Raf-Mek-Erk1/2 modules can signal from multiple cellular compartments (Figure 1*C*), including the late endosomes (M1) (23), caveolae (M2) (24), and the Golgi apparatus (M3) (25). Ras family GTPases may control, in part, the localization of each module. These GTPases carry autonomous address information at the carboxy-terminus specified by a pattern of methylation, prenylation, and palmitoylation that controls the targeting of the protein to a distinct membrane domain (11). The scaffold associated with the kinase cascade may also control compartmentalization. CNK contains a pleckstrin homology domain that mediates association with membrane phosphoinositides, which may mediate the association of insect Raf with membrane fractions (17, 26). KSR carries a cysteine-rich motif that can mediate membrane association, perhaps through interactions with phosphatidylserine (27). Finally, MP-1, a potential scaffold for MEK1 and MEK2, is required for the localization of ERK1/2 to late endosomes via an interaction with the resident endosomal protein p14 (23).

From this brief analysis of signaling through the ERK1/2 kinase cascade, we conclude that compartmentalization is a fundamental component of signaling

network design. In these compartments, signaling is organized and local environmental factors exert control. An important strategy for understanding signal transduction in cells, therefore, will be to devise methods to probe the functionality of signaling networks in the compartment where they normally reside.

PROFILE OF A SIGNALING COMPARTMENT

Many different compartments in the cell collect signaling units of one type or another. One compartment that has received a lot of attention in the signaling field is the plasmalemmal caveolae (28). Originally studied as regions of plasma membrane specialized for internalizing molecules in endothelial cells (29), caveolae are enriched in a variety of signaling molecules that are functionally linked to specific signaling cascades (30). We will use caveolae to illustrate how cellular compartments that contain signaling networks can be used to probe the functionality of these networks as they exist in the cell.

Caveolae as a Compartment

Cell compartments have a distinctive morphology and dynamics that are important for understanding the functionality of the signaling units they contain. Like all compartments, caveolae are constructed and maintained by specialized cellular machinery and exhibit characteristic behaviors that define their lifetime functions. They typically are recognized as flask-shaped membrane invaginations (31) decorated with a coat protein called caveolin-1 (32) and are best known as endocytic organelles that internalize specific classes of molecules. There appear to be two distinctive modes of internalization (Figure 2, see color insert). Some caveolae (Type 1) invaginate much the same as clathrin-coated pits do and pinch off from the membrane using dynamin to complete the fission step (33, 34). These caveolae are able to travel to the interior of the cell. Other caveolae (Type 2) become deeply invaginated to the point where they are functionally sealed off from the extracellular space but remain associated with the plasma membrane. These caveolae open and close without ever leaving the vicinity of the cell surface. Type 1 and Type 2 caveolae can be distinguished by their ligand internalization patterns. For example, uptake of folate by the GPI-anchored folate receptor involves caveolae that open and close in a $\sim$1 hr cycle, without ever leaving the vicinity of the plasma membrane, by a process called potocytosis (35). Internalization of SV40 virus, by contrast, depends on caveolae that pinch off from the plasma membrane and travel to the cell interior (36). Inhibiting either PKCα (36, 37) or tyrosine kinase activity (36, 38) blocks both types of internalization, although uptake by coated pits is unaffected.

The vesicles produced by caveolae (39) during endocytosis (cavicles) are impossible to identify by EM unless they are loaded with recognizable cargo. The introduction of caveolin-GFP (green fluorescent protein) has made it possible for the first time to study caveolae membrane traffic in detail. Unexpectedly, three

different kinds of traffic can be detected in tissue culture cells. The most common pattern (up to 75%) is a sessile behavior where the caveolin-GFP positive membrane appears to be firmly anchored at the cell surface (40). Sessile caveolin-GFP is also concentrated at the cleavage furrow of dividing cells (41). Relatively immotile caveolin-GFP is the expected behavior of a Type 2 caveola (Figure 2). A second behavior (Figure 2) is a rapid bidirectional, microtubule-dependent movement of caveolin-GFP positive vesicles (cavicles) between the center of the cell and the cell surface (39). This movement most likely corresponds to Type 1 caveolae that have budded from the membrane. In the case of CHO cells, cavicles appear to be traveling to the recycling endosome (42), although it is not clear if they fuse with this compartment. By contrast, cavicles carrying SV40 virus travel to a special endocytic compartment called the caveosome (36). The third type of movement detected with caveolin-GFP is the projection and retraction of fine tubular elements that can extend from the plasma membrane to the center of the cell (Figure 2). Recently these tubules have been captured in EM images of cells internalizing protein A-gold bound to prions (43). Because prions are also concentrated in flask-shaped caveolae (44), the tubular elements, designated Type 3 caveolae (Figure 2), may be derived from either Type 1 or 2 caveolae. To the extent that caveolin-GFP marks caveolae and cavicles, there appears to be a high degree of plasticity to the movement of caveolae-derived membranes. There are even instances in which entire sheets of caveolae membrane appear to internalize en masse to form internal, endosome-like structures (39), which may be how certain bacterial pathogens are internalized (45).

Isolating the Compartment

A principal tool for studying network organization in cellular compartments is cell fractionation. In the case of caveolae, it is relatively easy to isolate them from tissue culture cells. There are four methods in general use today. The first, and by far the most widely used, takes advantage of the detergent insolubility of caveolae membranes in combination with their light buoyant density on sucrose gradients (46). Triton X-100 insoluble, light membrane fractions can be prepared either from isolated plasma membranes or from the whole cell. The second method is an adaptation of the first in which the Triton X-100 is replaced with 500 mM sodium carbonate, pH 11 (47). Two other methods use neither detergents nor carbonate. One depends on sonication to break isolated plasma membrane into small pieces that are separated on the basis of their buoyant density (48). The other uses cationized silica to purify caveolae from isolated plasma membranes by homogenization, density gradient centrifugation, and, in some cases, immunoadsorption (49). The four methods do not yield exactly the same fraction of membranes, which can be an important issue when studying signal transduction.

To preserve the functionality of signaling units in their naturally organized state, the isolation procedure has to produce a "live" compartment. Therefore,

isolation needs to be rapid enough to retain the molecular composition of the compartment at the time a signaling pathway may be activated. Neither the Triton X-100 insolubility (50, 51) nor the pH 11, sodium carbonate method for isolating caveolae meet this requirement, because each is known to extract molecules that are native to caveolae. For example, Triton X-100 removes native prenylated proteins (51), and carbonate removes GPI anchored proteins (47). Triton X-100 has the added liability that it inactivates signaling molecules concentrated in caveolae (50). No isolation procedure yields a pure compartment. Isolated caveolae fractions, however, can yield real time in vivo and in vitro information about the dynamics of signaling networks that congregate in this compartment (see below).

Compartmentalization of Signaling Molecules

The first step in studying signaling compartments is to identify the resident signaling units. The evidence that caveolae are enriched in signaling units comes from three major sources. The first is cell fractionation. As soon as it was possible to obtain partially purified fractions of caveolae, many investigators found that signaling proteins such as receptor and nonreceptor tyrosine kinases, PKC, heterotrimeric G proteins, G-protein coupled receptors, eNOS, etc. were highly enriched relative to the plasma membrane. These early studies also established that signaling lipids like ceramide (52) and GM1 ganglioside (53) were enriched in caveolae. There are now hundreds of reports in which cell fractionation has been used to document that caveolae are enriched in a variety of different signaling molecules. Another important source of information has been the identification of signaling molecules that interact with caveolin-1. Using a combination of two-hybrid screen, immunoprecipitation, and various in vitro interaction techniques, more than 30 different signaling proteins and lipids have been identified that interact with caveolin-1 (54). Even though in many cases the exact function of these interactions remains to be established, an interaction with caveolin-1 is a good indicator that the molecule was in caveolae at the time of the experiment. Another source of information is light and electron microscopy. Immunofluorescence and immunogold probes have been very useful methods for showing that signaling molecules like Rho (55), Rac (56), H-Ras (57), PDGF receptor (24), ERK1/2 (24), PKCα (37), the PKC substrate SDR (58), and eNOS (59), to mention just a few, are concentrated in caveolae relative to other regions of membrane. In addition, histochemical methods have shown that second messengers like cAMP (60) and calcium (61) are concentrated in caveolae, indicating that the molecular interactions involved in regulating these signaling intermediates are functionally organized in this domain.

Functional Signaling Units in a Compartment

The molecular composition and known endocytic functions of caveolae originally suggested four types of signaling activities that might originate from this compartment: a) activation of tyrosine kinases, b) transduction of mechanical signals, c) regulation through second messengers, and d) the formation of chemical synapses

between neuronal and non-neuronal cells (62). Here we focus the discussion on three of these activities.

Receptor tyrosine kinases such as the PDGF receptor (24), the EGF receptor (63), and insulin receptor (64) have been localized to caveolae using cell fractionation, immunocytochemistry, or caveolin-1 interaction. Investigators have used several different experimental protocols to show that these tyrosine kinases are linked to signaling units in caveolae. One successful approach demonstrates that these receptors are coupled to other signaling molecules in caveolae in vivo. For example, binding of PDGF to PDGFR in caveolae stimulates the phosphorylation of multiple caveolar substrates (65) and silences EGFR phosphorylation in response to EGF (66). By contrast, EGF causes the recruitment of Raf-1 kinase to caveolae where it is activated (67), and it stimulates the local generation of inositol trisphosphate from a pool of caveolar phosphatidylinositol 4,5-bisphosphate (PtdIns 4,5-P_2) (68). The general protocol for these experiments is to expose the cells to the growth factor, prepare caveolae fractions, and assay for changes that occur in this membrane fraction but not in noncaveolae fractions.

An extension of this method is to assay for the presence of functional signaling units in isolated caveolae. The first test of this technique found that PDGFR was functionally linked to the activation of the MAP kinase ERK1/2 in isolated caveolae (24). The interaction of as many as 11 different molecules (PDGF, PDGFR, SOS, Ras, Raf-1, Grb2, SHC, 14–3-3, MEK-1, and ERK1/2) can be involved in activating ERK, so all the members of this signaling unit must be preorganized in the caveolae membrane, because nothing else was added to the preparation except PDGF. Indeed, immunoblotting has documented that many of these molecules are enriched in caveolae fractions (65).

Finding functional signaling units in caveolae fractions indicates that the isolated compartment can retain the cellular complexity necessary to study the natural switching and branching that occurs between signaling units in the living cell. Recent studies on activation of eNOS support this reasoning (69). eNOS is targeted to caveolae by an N-terminal acylation motif (59) and in this location can be activated by a number of different humoral and mechanical stimuli including estradiol, bradykinin, VEGF, HDL, isometric vessel contraction, and shear stress. The linkage between the stimulus and the activation of eNOS depends on the interaction of many cofactors and connectors, including nonreceptor tyrosine kinases, calcium, heterotrimeric G proteins, PI3 kinase, Akt kinase, ERK1/2, PKC, PKA, calmodulin, and HSP90. Many of these molecules and ions have been localized to caveolae. More important, the connectivity between these molecules is preserved in isolated caveolae. Incubation of isolated endothelial cell caveolae in the presence of estradiol (70), bradykinin (70), acetylcholine (70), or HDL (71) all stimulate eNOS enzymatic activity (Figure 3*A*), although these ligands have no effect on isolated noncaveolae membrane (Figure 3*B*). Thus, the natural organization of this signaling pathway is preserved so well that caveolae eNOS can be activated by four different ligands, each binding a receptor that is wired to eNOS through a distinct set of connecting molecules (72).

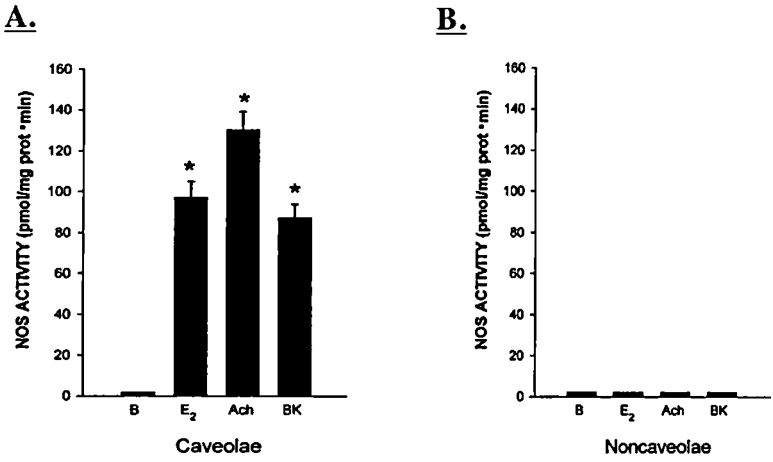

Figure 3 The eNOS signaling unit is located in caveolae. L-[^{3}H]-arginine conversion to L-[^{3}H]-citrulline was measured in caveolae (*A*), and noncaveolae (*B*) membrane fractions isolated from endothelial cells by the method of Smart et al. (48) and incubated in the absence (B) or presence of 10^{-8} M estradiol (E_2), 10^{-6} M acetylcholine (Ach), or 10^{-6} M bradykinin (BK). Values are mean +/− SEM (n = 4–6), $^*p < 0.05$ relative to basal. [Modified from (70).]

Probing the functionality of signaling units in isolated compartments will uncover unexpected molecular connections that will need to be verified in the living cell. A major technical advance that makes this possible is the development of designer fluorescence resonance energy transfer (FRET) probes capable of detecting specific signaling pathways in live cells (73). The typical probe is a chimeric protein consisting of a donor and an acceptor GFP, which have matched overlapping excitation and emission spectra, connected together by a sensor that is designed to bind a specific ionic or molecular intermediate in a signaling pathway (74). When the sensor binds the molecule or ion of interest it undergoes a conformational change that adjusts the distance between the two GFPs (e.g., cyan fluorescent protein and yellow fluorescent proteins). When the two GFPs are close together, intermolecular FRET occurs. Therefore, the emission ratio before and after cell stimulation is a relative measure of how much of the signaling molecule or ion is in the vicinity of the probe. If the probe is targeted to a cell compartment, then the probe will record signal transduction at that location.

The calcium sensor yellow cameleon is an example of a FRET probe that has been successfully used to monitor signal transduction from caveolae in living cells (75). There is considerable evidence that caveolae contain the molecular machinery for sensing [Ca^{2+}] (76, 77). Yellow cameleon was used to monitor the dynamics of [Ca^{2+}] in endothelial cells. To do this, the cameleon was targeted either to the cytoplasm, the plasma membrane, or caveolae. The internal ER Ca^{2+} stores were

then depleted and the relative [Ca^{2+}] detected by each cameleon from the respective sites was recorded as the concentration of extracellular Ca^{2+} was increased. The comparative response of the probe to being in the three different locations (i.e., compartments) indicates that caveolae are preferred sites of Ca^{2+} entry and that the entering Ca^{2+} is linked to the activation of eNOS. FRET-based probes promise to be extremely useful for mapping signaling networks in live cells.

Spatial and Temporal Organization of Signal Transduction

Compartments naturally restrict their special functions to the region of the cell where they are located. Therefore, compartmentalization spatially organizes signal units in the cell. Caveolae again provide a dramatic illustration of this point. In addition to containing the molecular machinery that controls Ca^{2+} entry, caveolae also contain the signaling molecules that regulate Ca^{2+} release from the ER (78). Ca^{2+} is released from the ER when endothelial cells are incubated in the presence of ATP (Figure 4, see color insert), and Ca^{2+} sensitive dyes show that sites of release colocalize with a subpopulation of caveolae on the cell surface (arrows, Figure 4*A*.1 and 4*A*.4). Because the ER is uniformly distributed beneath the plasma membrane, the other caveolae either do not contain the same sets of signaling units or the units they hold are inactive. Apparently not all the caveolae in the cell are the same, which implies that signal transduction from caveolae is spatially restricted both by the physical location of the caveolae and whether the signaling units are active. Caveolae will relocate to the trailing edge of migrating cells (79, 80). These caveolae contain active signaling machinery and ATP now stimulates Ca^{2+} release exclusively from ER at the trailing edge of the cell (arrow, Figure 4*B*.1 and 4*B*.4). Compartmentalization, therefore, is an important mechanism that cells use to carry signaling units to different locations in the cell.

Three things are necessary for compartmentalized signal transduction. First, the unit molecules must be in the right compartment; otherwise, they cannot connect to the proper signaling molecules. Second, the molecular ecology of the compartment must be able to support the connectivity between unit molecules and their downstream targets. Finally, the compartment must be in the right location at the right time. Studies of signal transduction from caveolae experimentally verify each of these principles.

Several different molecular addresses have been identified that direct molecules to caveolae, including the acylation motif of eNOS (59, 81), the second cysteine-rich region of the EGF receptor (82), and the transmembrane domain of influenza HA (83). eNOS lacking the acylation motif does not localize to caveolae and is disconnected from its normal signaling units (59, 70). Targeting to the proper compartment, therefore, is essential for normal signaling. On the other hand, if a signaling molecule is inappropriately targeted to caveolae, it may become connected to the wrong signaling units. In a recent test of this idea, oxytocin receptors (OTR) expressed in MDCK cells that are excluded from caveolin-rich membrane

domains inhibited cell growth in response to oxytocin. By contrast, when OTR was targeted to caveolae, oxytocin stimulated cell proliferation (84). The apparent cause of the disparate downstream effects was a difference in the EGFR/Erk activation patterns that occur in the two locations owing to OTR interacting with different signaling intermediates (85). Moreover, stimulation of EGFR phosphorylation was transient when OTR was in caveolae but prolonged when in noncaveolae membranes, which agrees with the finding that activated EGF receptors rapidly move out of caveolae membranes (63). The results are also consistent with the recent finding that TGFβ signaling depends on whether the TGFβ receptor is in caveolae or clathrin coated pits (86).

Molecular ecology refers to the environment created by the collective interactions of compartmental ions, lipids, proteins, carbohydrates, etc. A significant molecular component of a membrane compartment is the lipid bilayer, and for caveolae, the operative lipid is cholesterol (32, 87). Removal of cholesterol leads to the disintegration of caveola structure (87) and a loss of the ability of the domain to internalize molecules (88). Numerous studies have documented that removal or sequestration of cholesterol alters signal transduction from caveolae (57). Changes in caveolae cholesterol sometimes enhance signal transduction (89) and other times inhibit it (90). Presumably, cholesterol is required to maintain the characteristic phase properties of the caveolae membrane (91), which is essential for the proper organization and function of signaling units targeted to this compartment.

We discussed earlier how caveolae move around in cells and carry signaling machinery to different locations. This behavior raises the possibility that migratory compartments can acquire distinctive sets of signaling units at different locations in the cell or use resident units to connect to new downstream targets. Differential signaling from mobile caveolae appears to occur in migrating fibroblasts (56, 92). Integrins are membrane receptors for extracellular matrix proteins, such as fibronectin, that function to mediate cell adhesion and modulate signal transduction from growth factors (93). One of the signaling events that integrins control is the movement of activated Rac1 to the plasma membrane. Rac1 binds preferentially to membranes from adherent cells compared to those from suspended cells. Both Rac1 and Rho A are enriched in the caveolae fraction from unstimulated cells, but their concentration in the domain markedly increases when cells are exposed to PDGF (55). Moreover, recruitment of Rac1 is blocked when membranes are depleted of cholesterol (56). Unexpectedly, integrins regulate Rac1 recruitment to the plasma membrane by controlling whether caveolae are at the cell surface. Cells adhered to integrins have many caveolae on the surface, but within minutes after they are released, the caveolae internalize and migrate to the center of the cell. In response to the loss of caveolae from the plasma membrane, Rac1 recruitment no longer occurs and the activation of Pak is blocked. Apparently the transfer of caveolae relocates the Rac1 binding sites, leading to inactivation of downstream signals. Therefore, the movement of a compartment changes the response of a whole constellation of signaling molecules to their normal stimulus.

CONCLUSION

This analysis of caveolae illustrates how signal transduction is organized in a compartment that controls important spatial and temporal parameters necessary for fidelity in intracellular signaling. There are strong indications that other cellular compartments attract specific sets of signaling molecules and modules, so most likely, compartmentalization is a general way cells organize signaling networks. Some of the questions that emerge from the current analysis include (*a*) How can the same compartment contain operationally different sets of signaling molecules? (*b*) How do the different molecular ecologies of compartments affect the input and output signals of the same signaling unit? (*c*) What are the rules for targeting signaling units to specific compartments? (*d*) Are interacting sets of signaling units always in the same compartment of each cell type, or do they move around? (*e*) What are the thermodynamic rules for how signaling networks are superimposed on to the architecture of the cell? Clearly, a systems biology approach to understanding cell structure and function will require answers to these and many similar questions.

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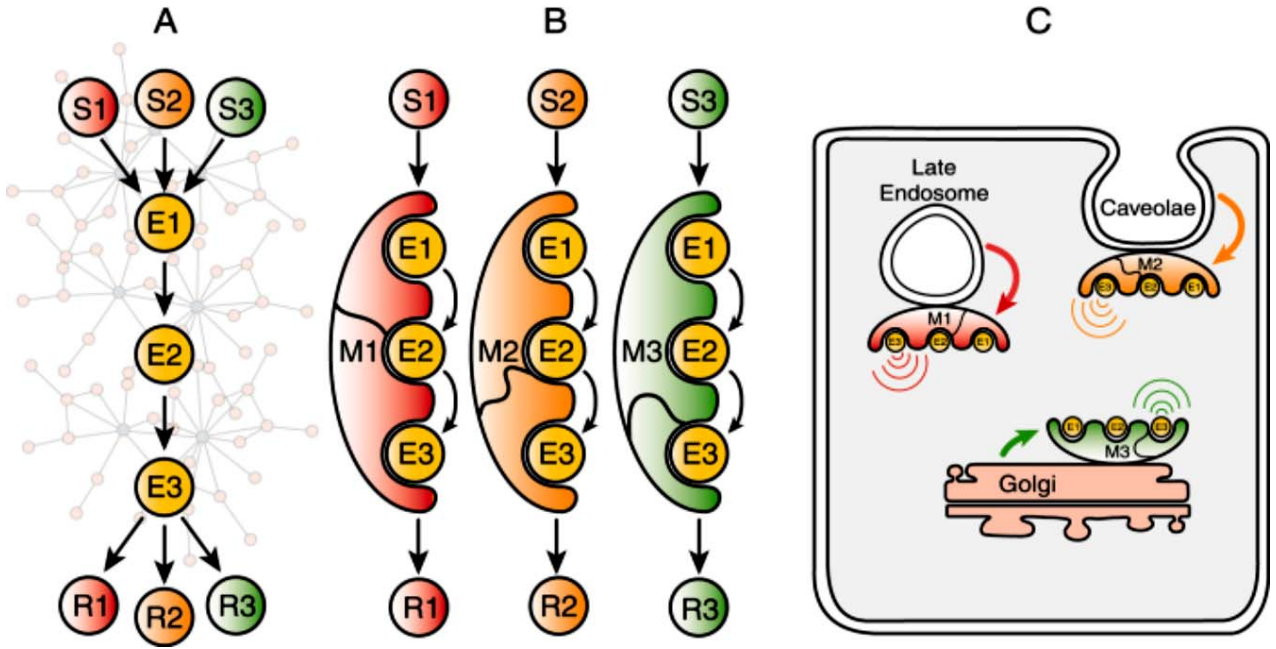

Figure 1 Schematic representation of how high fidelity signal transduction machines can be generated through modular organization of commonly engaged signaling proteins. (*A*) Representation of the biochemical relationships in a regulatory network with multiple distinct inputs (stimuli S1, S2, and S3) and outputs (responses R1, R2, and R3) that propagate through a common core enzymatic cascade (enzymes E1, E2, and E3). (*B*) Non-enzymatic accessory proteins (M1, M2, and M3) functionally segregate the core enzymatic cascade into separate modules with discrete input/output relationships. (*C*) Selective compartmentalization may restrict/facilitate coupling of spatially discrete stimuli to distinct signaling modules thereby generating fidelity among stimulus/response pathways.

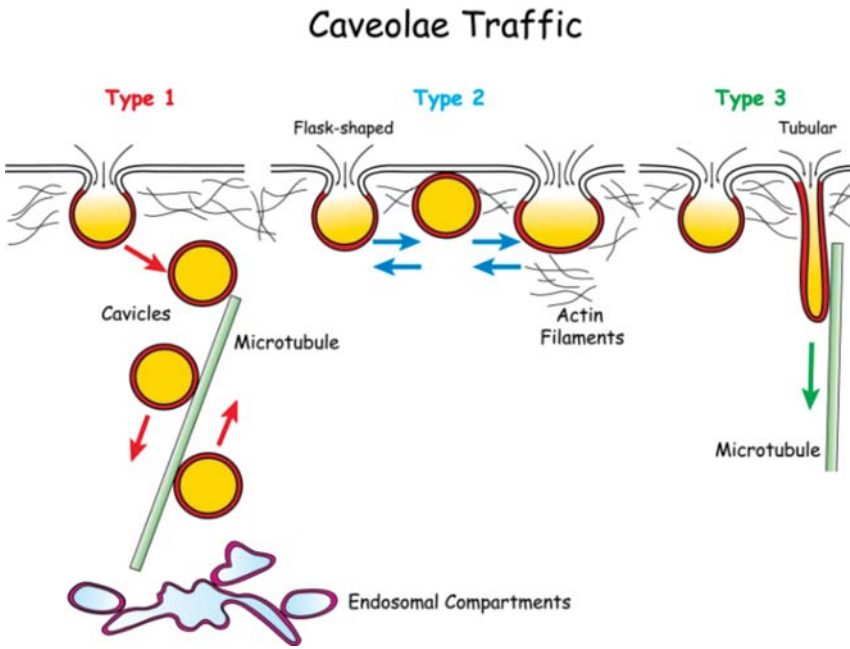

Figure 2 Multiple pathways of caveolae traffic. Type 1 caveolae are able to invaginate and bud from the membrane, probably in a dynamin-dependent process (33, 34). The vesicles that form, called cavicles, are able to travel on microtubules to various endosomal compartments. Cavicles also can travel from endosomes to other places in the cell. Type 2 caveolae invaginate and seal off from the plasma membrane but are retained at the surface by the actin cytoskeleton. We imagine that type 3 caveolae begin as membrane invaginations similar to the other types but then get caught on microtubules and become stretched by microtubule motor activity into tubules. (Diagram adapted from 39.)

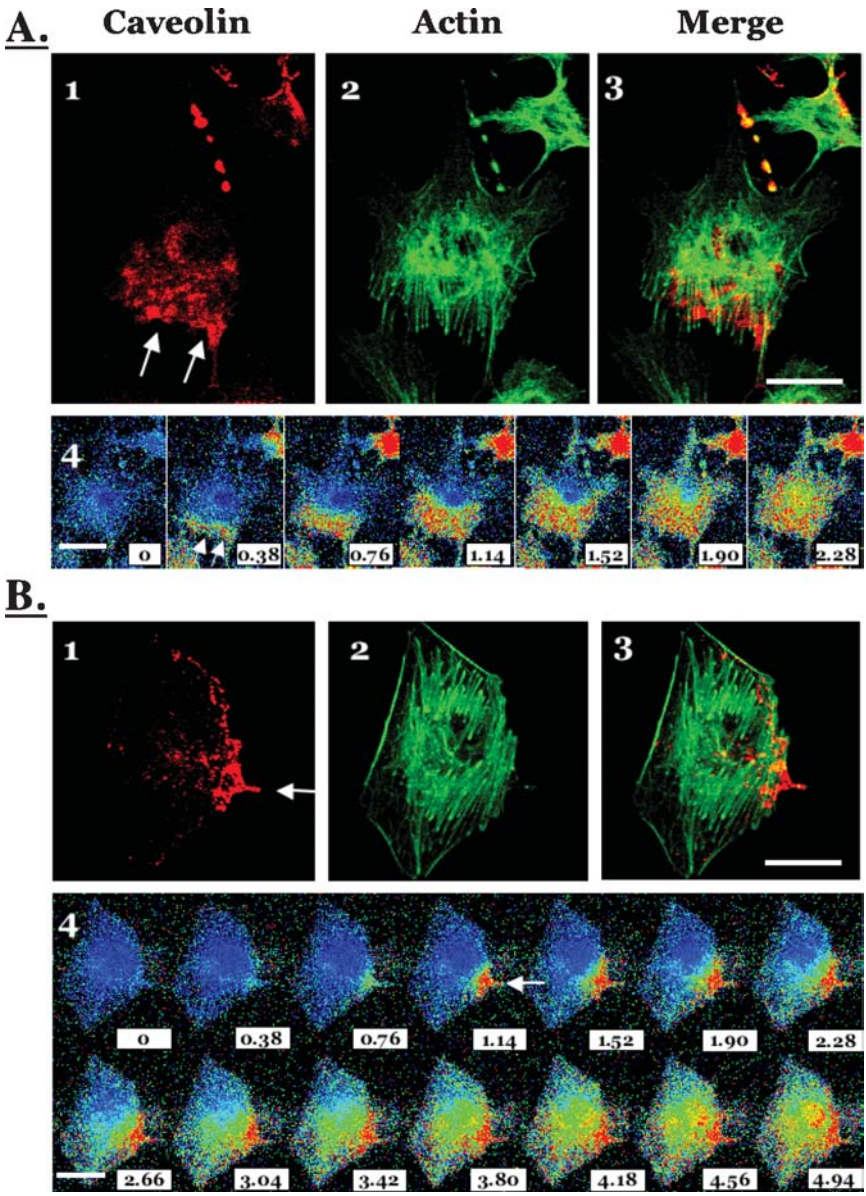

Figure 4 See legend on next page

Figure 4 Polarization of caveolae leads to polarization of ATP-dependent release of Ca^{2+} from the ER. Primary cultures of endothelial cells were either cultured on coverslips (*A*) or induced to migrate to the left by subjecting the cells to a fluid shear for 24 hr (*B*). Both sets of cells were loaded with the Ca^{2+} sensing dye Indo-1 (5 μM) before being incubated in the presence of either 0.5 M ATP (*A*) or 2 μM ATP (*B*). Images were taken at 0.38 sec intervals to visualize Ca^{2+} release (panel 4). At the end of the recording, the coverslip was fixed and processed to localize caveolin-1 (panel 1) and actin (panel 2). The merge of 1 and 2 is shown in 3. Notice in *A* that not all the caveolin-positive sites are active in ER Ca^{2+} release, indicating that caveolae are heterogeneous in their ability to transmit signals to the ER. Arrows indicate areas of caveolin-1 positive membrane that were active in stimulating Ca^{2+} release from the ER. Bar, 20 μM. See Reference 79 for details.

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