Compartmentalized Ras/MAPK Signaling

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Abstract

Signal transduction down the Ras/MAPK pathway, including that critical to T cell activation, proliferation, and differentiation, has been generally considered to occur at the plasma membrane. It is now clear that the plasma membrane does not represent the only platform for Ras/MAPK signaling. Moreover, the plasma membrane itself is no longer considered a uniform structure but rather a patchwork of microdomains that can compartmentalize signaling. Signaling on internal membranes was first recognized on endosomes. Genetically encoded fluorescent probes for signaling events such as GTP/GDP exchange on Ras have revealed signaling on a variety of intracellular membranes, including the Golgi apparatus. In fibroblasts, Ras is activated on the plasma membrane and Golgi with distinct kinetics. The pathway by which Golgi-associated Ras becomes activated involves PLCγ and RasGRP1 and may also require retrograde trafficking of Ras from the plasma membrane to the Golgi as a consequence of depalmitoylation. Thus, the Ras/MAPK pathway represents a clear example of compartmentalized signaling.

INTRODUCTION

Ras: rat sarcoma viral oncogene

MAPK: mitogen-activated protein kinase

ER: endoplasmic reticulum

GEF: guanine nucleotide-exchange factor

GAP: GTPase-activating protein

Signal transduction involves the transmission of biochemical information from one part of the cell to another. The best-studied signaling paradigms involve conveying information from the extracellular environment into the cell, often to the nucleus. Because many of the receptors that sense extracellular ligands are transmembrane proteins that traverse the plasma membrane, this compartment has been considered to be the primary platform upon which signaling complexes are assembled. This emphasis on the plasma membrane has certainly been true for T lymphocyte signaling, for which the antigen receptor initiates a nexus of signaling involving kinases, adapter proteins, scaffolds, GTPases, and phospholipases, all of which are generally considered to function at the inner leaflet of the plasma membrane. The advent of genetically encoded fluorescent probes for signaling events have enabled spatiotemporal analysis of signaling in living cells. Using these methods, signaling events previously presumed to be restricted to the plasma membrane have been observed on intracellular membranes. Among the signaling pathways that have provided the biggest surprises when analyzed spatially in living cells is the Ras/MAPK pathway. In addition to the plasma membrane, Ras and/or MAPK signaling has now been observed on endosomes, the endoplasmic reticulum (ER), the Golgi apparatus, and mitochondria. Ras signaling in lymphocytes has provided perhaps the biggest surprise of all because the primary platform for signaling appears to be the Golgi apparatus. Subcellular compartmentalization of signaling, such as that regulated by Ras, provides one explanation for the apparent complexity of signaling outputs elaborated by individual signaling molecules. In this review, we examine the current evidence for compartmentalized signaling, focusing on the Ras/MAPK pathway and emphasizing whenever possible evidence provided by the study of lymphocytes.

Ras BIOLOGY

The GTPase

The great fascination of cell biologists with Ras stems from the early association of the *ras* gene with cancer (1). Once the function of Ras as a GTPase was discovered, it became the prototypical monomeric GTPase. Much of the biochemistry that applies to the large superfamily of small GTPases was first elucidated by the intensive study of Ras. Ras proteins are molecular switches that cycle between inactive, GDP-bound, and active, GTP-bound, forms. Signal-induced conversion of the inactive to active state is mediated by guanine nucleotide-exchange factors (GEFs) that stimulate the exchange of GDP for GTP. This is accomplished by catalyzing the release of GDP from the guanine nucleotide binding pocket. Once it is nucleotide free, Ras will bind GTP because GTP is tenfold more abundant in cellular cytosol than is GDP. GTP binding induces Ras activation by causing a marked conformational change of the so-called switch I and switch II regions (2). These regions contribute to the effector-binding domain that engages downstream signaling elements only when the protein is in the GTP-bound state. The activation state of Ras is self-limited by the intrinsic GTPase activity of the protein. However, Ras, like most signaling GTPases, is a poor enzyme, with a K_{cat} of 2.3×10^{-4} sec^{-1} (3). The catalytic activity of Ras is greatly accelerated by a class of accessory proteins known as a GTPase activating proteins (GAPs). GAPs afford a critical level of regulation, allowing signaling to proceed for a relatively brief interval. The study of the biological function of Ras has been greatly facilitated by the availability of constitutively active as well as dominant-negative forms. The former are GTP-bound and oncogenic (see below), and the latter mimic the nucleotide-free transition state such that they have a high affinity for GEFs and stoichiometrically sequester these regulatory molecules (4).

The Oncogene

Ras genes encoded by rat sarcoma viruses, v-H-ras and v-K-ras, were among the first oncogenes to be recognized (5). These viral genes proved to be mutant forms of cellular protooncogenes (6–8). The commonly occurring mutations that render Ras oncogenic (at codons 12, 13 and 61) are those that make the GTPase insensitive to the action of GAPs and thereby lock it in the GTP-bound, active state (1, 9). Activated Ras alleles constitute the oncogene most frequently associated with human carcinomas (1), highlighting the great impact of Ras on human health. Mammalian genomes encode three *ras* genes that give rise to four gene products, N-Ras, H-Ras, K-Ras4A, and K-Ras4B, that are expressed ubiquitously, although isoform ratios vary from tissue to tissue. K-Ras4A and K-Ras4B are splice variants of the *kras* gene that use alternative fourth exons. Thirty percent of all human cancers harbor mutant *ras* genes. Tumors differ both in the isoforms associated with the disease and in the incidence of mutations of that isoform. For example, whereas 90% of pancreatic adenocarcinomas are associated with an oncogenic *Ras* mutation that is invariably in the *kras* gene, only 10% of bladder carcinomas harbor *Ras* mutations, and these occur in the *hras* gene (10).

The Protooncogene: Ras and T Cell Function

As an oncogene, Ras regulates pathways that lead to cellular proliferation and survival. However, proliferation is but one of many cellular functions that can now be ascribed to protooncogenic Ras. Among the cells in which the role of protooncogenic Ras has been most extensively studied are lymphocytes (11). Indeed, engagement of the T cell receptor (TCR) was the first physiologic stimulus shown to activate Ras (12). Subsequent studies have demonstrated that Ras is required for thymocyte development, T cell proliferation, and IL-2 production (13). Transgenic mice that express a dominant-negative form of Ras in the thymus have a defect in positive but not negative selection of thymocytes (14). Conversely, constitutively active Ras expressed in RAG-2-deficient thymocytes promotes the expansion of double-negative cells and their transition to double-positive cells (15). Constitutively active Ras expressed in T cells also results in overexpression of the early activating antigen, CD69, on the cell surface as well as activation of AP-1 in the nucleus. Targeted disruption of the Ras exchange factor Ras guanine nucleotide-releasing protein 1 (RasGRP1) results in a defect in thymocyte differentiation and decreased mature CD4 and CD8 cells, supporting the idea that Ras signaling downstream of the TCR is critical for thymocyte development (16). Autoimmunity that develops in adult RasGRP1-deficient mice suggests that Ras signaling may also be required to maintain peripheral T cell tolerance (17). Recent experiments with a RasGRP1-deficient Jurkat T cell line and RasGRP1 siRNA in wild-type cells confirmed that this GEF is required for optimal antigen receptor–triggered Ras-Erk activation and that this pathway is augmented by phosphorylation by atypical protein kinase Cs (PKCs) of threonine residue 184 of RasGRP1 (18).

RasGRP1: Ras guananine nucleotide-releasing protein 1

Ras PROCESSING AND TRAFFICKING

Ras proteins are localized on the cytosolic leaflet of cellular membranes (19, 20), and this localization is believed to be absolutely required for biological activity (21). Ras proteins are not intrinsic membrane proteins in that they lack signal sequences and hydrophobic membrane-spanning domains. Rather they are synthesized as hydrophilic proteins on free polysomes in the cytosol and targeted posttranslationally to cellular membranes by virtue of a series of modifications that include prenylation, proteolysis, and carboxyl methylation (22). Ras GTPases are the founding members of a large class of proteins that terminate in a CAAX motif, in

which C is cysteine, A is usually but not always an aliphatic amino acid, and X is any amino acid (23). The CAAX sequence is recognized by one of two cytosolic prenyltransferases (24), farnesyl transferase (FTase) or geranylgeranyltransferase type I (GGTase I). The amino acid in the X position determines which prenyltransferase modifies the protein (S, M, A or Q for FTase and L for GGTase I). FTase catalyzes the addition of a 15-carbon farnesyl isoprenoid, whereas GGTase I catalyzes the addition of a 20-carbon geranylgeranyl isoprenoid to the CAAX cysteine via a stable thioether linkage (25). Once prenylated, the *S*-isoprenyl CAAX moiety becomes a substrate for Rce1, a protease that cleaves the AAX sequence (22, 26). The newly C-terminal prenylcysteine is then recognized by a third enzyme, isoprenylcysteine carboxyl methyltransferase (Icmt), that methylesterifies the carboxyl group (22, 23). Of these three modifications, only carboxyl methylation is reversible under physiologic conditions (27). The end result of these three modifications is to create a hydrophobic domain at the C-terminus that mediates membrane association. N-Ras, H-Ras, and Kras4A, but not K-Ras4B, are further modified by one or two palmitic acids just upstream of the farnesylcysteine. The Ras palmitoyltransferase was recently identified in a genetic screen of *Saccharomyces cerevisiae* as Erf2/4 (28).

CAAX processing was originally conceived of as a process that targeted nascent cytosolic Ras proteins directly to the plasma membrane. The discovery that Icmt is restricted to the ER (29), and the subsequent characterization of the other postfarnesylation processing enzymes Rce1 (30) and Erf2/4 (31) as similarly restricted, led to a reevaluation of Ras trafficking (**Figure 1**). CAAX processing alone is insufficient for association with the plasma membrane but is instead a mechanism for directing proteins to the ER, where they encounter Rce1, Icmt, and Erf2/4, the processing enzymes that further modify prenylated proteins (20). The subsequent transfer of Ras proteins from the endomembrane to the plasma membrane requires a second signal found in the C-terminal hypervariable regions of Ras proteins immediately adjacent to their CAAX motifs. For N-Ras, H-Ras, and K-Ras4A (the minor splice variant of the *kras* gene), this signal consists of one or two cysteine residues that serve as sites of palmitoylation. For K-Ras4B, the ubiquitous and predominant splice variant henceforth referred to as K-Ras, the signal consists of a polybasic region rich in lysine residues (32, 33). Whereas prenylation alone serves as a relatively weak membrane tether, palmitoylation markedly increases affinity for membranes and traps N-Ras and H-Ras in the membrane compartment. Palmitoylated CAAX proteins such as N-Ras and H-Ras are translocated by vesicular transport (20) that may involve the classical secretory pathway or a nonclassical pathway (34). In contrast, CAAX proteins with a polybasic second signal such as K-Ras take an alternate, poorly defined route to the plasma membrane.

Recently two groups independently discovered a retrograde pathway of trafficking of palmitoylated Ras isoforms (35, 36). In this pathway, mature N-Ras and H-Ras that have gained access to the plasma membrane are depalmitoylated and thereby lose sufficient affinity for membranes such that they partition into the cytosol. From there they regain access to the endomembrane, where they can be repalmitoylated. Thus, N-Ras and H-Ras undergo a palmitoylation/depalmitoylation cycle that regulates trafficking from the plasma membrane to the Golgi and back again. This plasma membrane/Golgi cycle may be generalized to a wide range of palmitoylated signaling molecules, which has led to speculation that the bidirectional traffic has a regulatory role in signaling (36). Recently, K-Ras has also been shown to traffic in a retrograde fashion to the Golgi apparatus in hippocampal neurons stimulated with glutamate (37) and after phosphorylation by PKC in the polybasic region to the outer mitochondrial membrane in a variety of cells, including Jurkat T cells

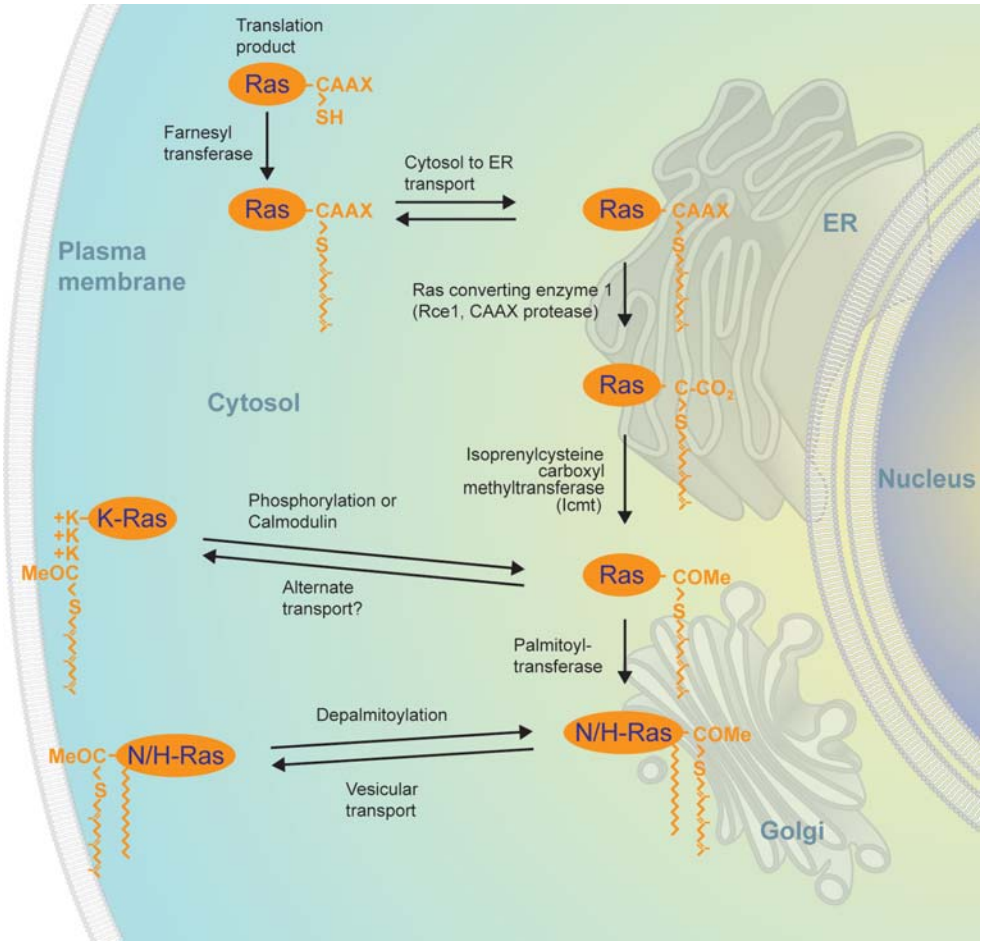

Figure 1

Ras processing and trafficking. Ras and other CAAX proteins are translated in the cytosol on free polysomes. Immediately posttranslationally, they become substrates for one of two cytosolic prenyltransferases. Prenylation targets the proteins to the ER, where they encounter the subsequent processing enzymes, Rce1 and Icmt. Once CAAX processing is complete, the pathways used by the various isoforms diverge. K-Ras is sent to the plasma membrane via an uncharacterized pathway and can be returned to the endomembrane following phosphorylation of the hypervariable region or through the action of calcium/calmodulin. In contrast, N-Ras and H-Ras are further processed on the Golgi by a palmitoyltransferase and then sent to the plasma membrane via vesicular transport. Retrograde traffic of N-Ras and H-Ras back to the Golgi occurs following depalmitoylation.

(M.R. Philips, unpublished data). Thus, although until recently mature Ras proteins were thought to be stationary, it is now clear that they traffic between cellular compartments. This revelation has made all the more prescient the question of spatiotemporal Ras signaling.

Ras SIGNALING

The Erk Pathway

The best characterized of the signaling pathways regulated by Ras is the MAPK pathway that proceeds through the MAPKs Erk1 and Erk2. This pathway is activated when

PTKR: protein tyrosine kinases receptor

GPCR: G protein–coupled receptor

CCV: clathrin-coated vesicles

any of several growth factors engage their cognate protein tyrosine kinase receptors (PTKRs). Ligation of PTKRs promotes their dimerization, which in turn allows cross-phosphorylation of tyrosine residues in their cytosolic domains catalyzed by the kinase that is intrinsic to that domain (38). These phosphotyrosine residues serve as docking sites for signaling molecules and adapter proteins that contain SH2 (Src homology region 2) or PTB (phosphotyrosine-binding) domains. Among these is the adapter protein Grb2 that binds constitutively to SOS through its SH3 domain. SOS (son of sevenless) is a GEF for Ras proteins. Thus, phosphorylation of PTKRs leads to the recruitment of SOS to the plasma membrane, where it can encounter Ras.

Once Ras is activated at the membrane, it recruits Raf-1, a serine/threonine kinase, to this compartment. The kinase activity of Raf-1 is activated when it associates with membranes through a complex set of regulatory events that are poorly understood (39). Once active, Raf-1 phosphorylates and activates MEK (MAPK/Erk kinase), a dual specificity tyrosine/threonine kinase, that in turn phosphorylates and activates Erk1 and Erk2. The Erk proteins are serine/threonine kinases that have numerous substrates, including cytosolic proteins like p90 ribosomal S6 kinase. Phospho-Erk forms dimers that are transported into the nucleus, where they phosphorylate the Ets family of transcription factors, including Elk-1. In this way the signal emanating from an extracellular growth factor is transmitted from the cell surface to the nucleus, and a transcriptional program is set in motion.

Other Pathways

The Raf-1/Erk pathway is but one of many that are regulated by Ras. Ras effectors are defined as proteins that bind Ras with a strong preference for the GTP-bound state. A more stringent definition requires that the function of the effector is modulated by the binding to GTP-Ras. The precise number of known Ras effectors is somewhat uncertain because it depends how rigorously the more stringent definition is applied, but it appears that there are at least ten types of proteins that are putative effectors (40). Besides Raf-1 and its homologs, the best-characterized effectors are phosphatidylinositol 3-kinase (PI3K) and members of a family of exchange factors for the small GTPase Ral, e.g., RalGDS. PI3K activates Akt and thereby promotes cell survival. Although the Raf-1/MAPK pathway has been considered to be the most important with regard to Ras-mediated transformation of rodent fibroblasts, recent studies have suggested that in human cells the RalGDS pathway is the most important in oncogenesis (41).

SIGNALING FROM ENDOSOMES

PTKRs and Associated Proteins

As described above, Ras links receptors such as PTKRs that traverse the plasma membrane with cytoplasmic effector pathways that regulate cellular function, including transcription. As such, it is intuitive to think of Ras signaling from the plasma membrane. Indeed, for the first two decades of the study of Ras, the plasma membrane was considered the only platform from which Ras could signal. The first indication that the plasma membrane may not be a unique platform for Ras/MAPK signaling came from the discovery that signaling complexes could be detected on endosomes (42, 43).

Investigators have appreciated for decades that receptors such as PTKRs, G protein–coupled receptors (GPCRs) and TCRs are subject to removal from the cell surface by endocytosis mediated by clathrin-coated vesicles (CCVs). This process was originally viewed as one of the major mechanisms for receptor desensitization. Internalized receptors are either further trafficked to lysosomes for degradation or recycled back to the plasma membrane (44). Among the proteins that regulate receptor internalization is c-Cbl. The Cbl family of proteins consists of c-Cbl, Cbl-b,

and Cbl-c, and these proteins function as E3 ubiquitin ligases (45, 46). c-Cbl monoubiquitinates a variety of receptors, including epidermal growth factor receptor (EGFR), in a ligand-dependent fashion and thereby targets the receptor for endocytosis and degradation via the proteosome and/or lysosome (47). An oncogenic, viral form of this protein, v-Cbl, interferes with the receptor degradation pathway and shunts receptors toward the recycling pathway (47), offering compelling evidence for the physiologic importance of endocytosis in downregulation of growth factor receptor signaling. Cbl is also a binding partner of Grb2, raising the possibility that, in addition to promoting receptor internalization, it influences Ras signaling (48). In the case of GPCRs, the β-arrrestins play a critical role in endocytosis by linking the receptor to elements of the clathrin coat (49). Among the GPCRs that are regulated by β-arrestin-mediated endocytosis are chemokine receptors on lymphocytes that regulate a wide array of lymphocyte function and serve as coreceptors for HIV (50).

T cell activation by specific antigen results in a rapid and profound internalization and degradation of the TCR (51). Since c-Cbl is required for T cell function, it has been suggested that, like PTKRs, TCRs may be downregulated by Cbl-induced endocytosis and degradation. Consistent with this idea, TCR surface expression is enhanced in mice deficient in c-Cbl, and this is associated with altered positive selection in the thymus (52). Mice lacking Cbl-b develop autoimmunity, suggesting that in vivo immune tolerance is controlled by signaling pathways in T cells that are constitutively suppressed by Cbl-b (53). These mice also exhibit reduced TCR downregulation, increased cytokine production, and uncontrolled proliferation in response to anti-CD3 antibodies. Cbl ubiquitination has been visualized at the immunological synapse, and Cbl recruitment was found to be antigen dependent (53).

As discussed above, the evidence for endocytosis as a mechanism for downregulating receptor signaling is strong. But there is another side to the story of endosomes and signaling. The first evidence for signaling on endosomes came from subcellular fractionation studies in which Shc, Grb2, mSOS, and phospho-Raf-1 were differentially observed on endosomes following stimulation with EGF or with insulin (42). Subsequently, the discovery that cells deficient in clathrin-mediated endocytosis were also deficient in Erk activation downstream of PTKRs provided compelling evidence that endocytosis could drive MAPK signaling rather than simply downregulate the pathway at the level of the receptor (54, 55). The most widely used method of blocking clathrin-mediated endocytosis has been to express the K44A dominant-negative form of the large GTPase dynamin that is believed to regulate scission of endosomes (54). Such studies have not been confined to PTKRs but have also been applied to GPCRs. Interestingly, K44A blocks signaling from GPCRs to the MAPK pathway but does not block signaling to other pathways such as adenylcyclase. Moreover, Erk activation was blocked, but GTP loading of Ras was not (56). These studies have prompted a reevaluation of the role of endocytosis in signaling and have led to the idea of signaling complexes on endosomes.

Several methods have been used to demonstrate that PTKRs remain active after internalization on endosomes. Unlike TGF-α, which dissociates from EGFR in early endosomes, EGF remains bound to its receptor, suggesting that ligands differ in their ability to sustain signaling on internalized receptors (57). Antiphosphotyrosine antibodies were used to reveal persistent phosphorylation of internalized EGFR (58). Sorkin and coworkers (59) demonstrated internalization of activated, CFP-tagged EGFRs by FRET using YFP-tagged Grb2 as a phosphotyrosine sensor. Importantly, neither the kinase activity nor the trafficking of EGFR was altered by fusion with GFP (60). Using fluorescence lifetime imaging as a sensitive read out of FRET, Wouters & Bastiaens (61) obtained

EGFR: epidermal growth factor receptor

FRET: fluorescence resonance energy transfer

YFP: yellow fluorescent protein

GFP: green fluorescent protein

RBD: Ras-binding domain

results similar to those of Sorkin et al. (59) by using GFP-tagged EGFR and microinjected antiphosphotyrosine antibodies conjugated with a FRET acceptor.

In addition to phosphorylated, active PTKRs, other upstream components of Ras signaling have been localized on endosomes, including Shc, Grb2, and SOS (42, 58, 59, 62–65). PLCγ1 has also been localized on endosomes (66, 67), an observation that may have implications for T cells because Ras activation in these cells is downstream of this enzyme (11). Ras itself was observed on endosomes using subcellular fractionation (68). Using GFP fusion proteins and live cell imaging, Ras has been localized to vesicles including endosomes (20, 62). Importantly, Sorkin and colleagues (62) have used the Ras-binding domain (RBD) of Raf-1 fused to YFP to reveal GTP-bound Ras on vesicles decorated with internalized EGF. Hancock and colleagues (69) used dominant-negative dynamin K44A to show that H-Ras but not K-Ras signaling was dependent on endocytosis. Activated GFP-H-Ras12V was associated with Rab5-GTP-induced macroendosomes, whereas activated GFP-K-Ras12V was not. Interestingly, GFP-Raf-1 was not observed on these structures.

Intersectin, an interesting adapter protein in clathrin-mediated endocytosis, binds mSOS, providing a direct link between the endocytic machinery and Ras activation. Intersectin contains two Eps15 homology domains with which it binds epsin that in turn interacts with AP-2 and clathrin and thereby recruits intersectin to clathrin-coated pits (70). Intersectin also has five tandem SH3 domains, two of which bind dynamim and synaptojanin, other components of the endocytic machinery (71). One of the remaining SH3 domains binds to mSOS (72), which can activate Ras on endosomes (73). Functional studies reveal that intersectin is involved both in the formation of endosomes (74) and in mitogenic signaling (75). Thus, intersectin lives up to its name as a molecule at the intersection of clathrin-mediated endocytosis and Ras signaling (76).

Because endosomes derive from the plasma membrane, and because signaling from the latter compartment does not cease once endosomes have formed, demonstrating unambiguously that signals emanating from endosomes drive pathways that are also driven from the plasma membrane has been difficult. Wang and colleagues (77) have taken a clever approach to this problem. They stimulated cells with EGF in the presence of a readily reversible EGFR kinase inhibitor and in the presence of monemsin, which blocks receptor recycling to the surface. After EGFR internalization, extracellular EGF was washed away, and signaling was subsequently permitted by washing away the EGFR kinase inhibitor. Under these conditions, Erk was phosphorylated and the cells received a survival signal, demonstrating effective signaling from endosomes.

Rap1

Whereas endosomes are not the primary compartment to which Ras proteins are targeted, these organelles are the primary location of Rap1, a closely related small GTPase (78). Rap1 is the GTPase most closely related to Ras and shares 100% of the effector domain amino acids known to contact the RBD of effectors (79). Rap1 has been implicated in a wide variety of cellular functions, including growth control and cell polarity (79). Although Rap1, like Ras, can bind to Raf-1, the ability of Rap1 to substitute for Ras in activating the MAPK pathway has been controversial (80). A recent genetic study in *Drosophila* indicated that Rap1 can indeed activate Erk in a Ras-independent fashion (81). Among the biological functions of Rap1 is the regulation of cellular adhesion, and within this category the best-studied pathway in which Rap1 is implicated is the regulation of integrins through "inside-out signaling" (82). Activated Rap1 stimulates lymphocyte function–associated antigen (LFA)-1-mediated T cell adhesion (83). The relevant effector for this pathway is RapL, a member of the RASSF family of tumor suppressors (84). RapL binds

to the cytoplasmic tail of the α chain of LFA-1, suggesting a direct link to integrin affinity modulation (85).

As with most Ras family GTPases, the greatest divergence in sequence between Ras and Rap occurs in the membrane-targeting regions of the C-terminus. Unlike Ras proteins that are farnesylated, Rap1 is geranylgeranylated. In both fibroblasts and T cells Rap1 is expressed predominantly on endosomes (78). Matsuda and colleagues (86) were the first to study the subcellular localization of Rap1 activation in living cells. They used an innovative FRET probe called RAICHU and concluded that Rap1 was activated in the perinuclear region of the cell, although the limited spatial resolution of their method did not reveal a particular organelle as the site of activation (86). Using a reporter for active Rap1 based on the RBD of the exchange factor RalGDS that has a 50-fold higher affinity for Rap1 than for Ras, Bivona et al. (78) observed Rap1 activation in fibroblasts stimulated with growth factor and Jurkat T cells stimulated through the antigen receptor only on the plasma membrane (**Figure 2**). The discrepancy in the location of activation reported by these two methods likely stems from the fact that the RAICHU Rap1 reporter was ectopically targeted with a K-Ras sequence, whereas the RalGDS-based probe was untargeted and therefore spatially unbiased. The activation of Rap1 at the plasma membrane of T cells was recently independently confirmed (87). Because Rap1 regulates the adhesive state of integrins that function at the plasma membrane, this is likely the site of activation of this GTPase. But if Rap1 functions at the plasma membrane, why is the bulk of the protein found on endosomes? Part of the answer is that Rap1 has multiple functions, and conditions under which Rap1 becomes active on endosomes may yet be found (88). Another possibility is that Rap1 on endosomes may serve as a storage pool analogous to the pool of integrins that is stored in the membranes of leukocyte granules. Consistent with this idea, the activation of Rap1 at the plasma membrane of Jurkat cells is dependent on endosome recycling (78).

Special Cases: Neural Cells

What is the advantage to the cell in assembling or maintaining signaling complexes in the Ras/MAPK pathway on endosomes? Aside from the obvious answer that multiple signaling platforms increase the complexity of signaling outputs, another possible answer is that signaling endosomes allow messages to be delivered over great distances within cells. Nowhere is the need for such long-distance delivery more evident than in neuronal cells. Neurotropin receptors at nerve terminals must signal for events that take place in the neuronal cell body and nucleus that can be up to a meter from the terminal. Simple diffusion through the cytosol of, for example, phosphorylated Erk does not seem to be a reasonable solution for such long-range signaling. We now clearly know that, in neuronal cells, signaling endosomes are transported in a retrograde fashion via microtubules and function to convey activated neurotropin PTKR from the site of activation in the nerve terminal to the perinuclear region of the cell body (89). Indeed, the term signaling endosome was coined in the context of neurotropin signaling (90). Early efforts at isolating signaling endosomes from PC12 cells indicated that nerve growth factor (NGF)-binding enhanced the internalization of phosphorylated TrkA into CCVs (90) and that these vesicles contained signaling complexes that included Shc, Ras, Raf-1, and Erk (91). Increased levels of activated Ras were detected on these CCVs, and isolated CVVs were capable of stimulating the phosphorylation of Elk-1, an endogenous substrate of Erk. More recently, experiments using a cell body chamber that isolates the cell body from the neuron extensions showed that phosphorylated TrkA (a neurotropin PTKR) accumulates in the neuron cell bodies (92). Within the immune system, dendritic cells have the longest distances to cover in cell signaling because in some anatomical locations

NGF: nerve growth factor

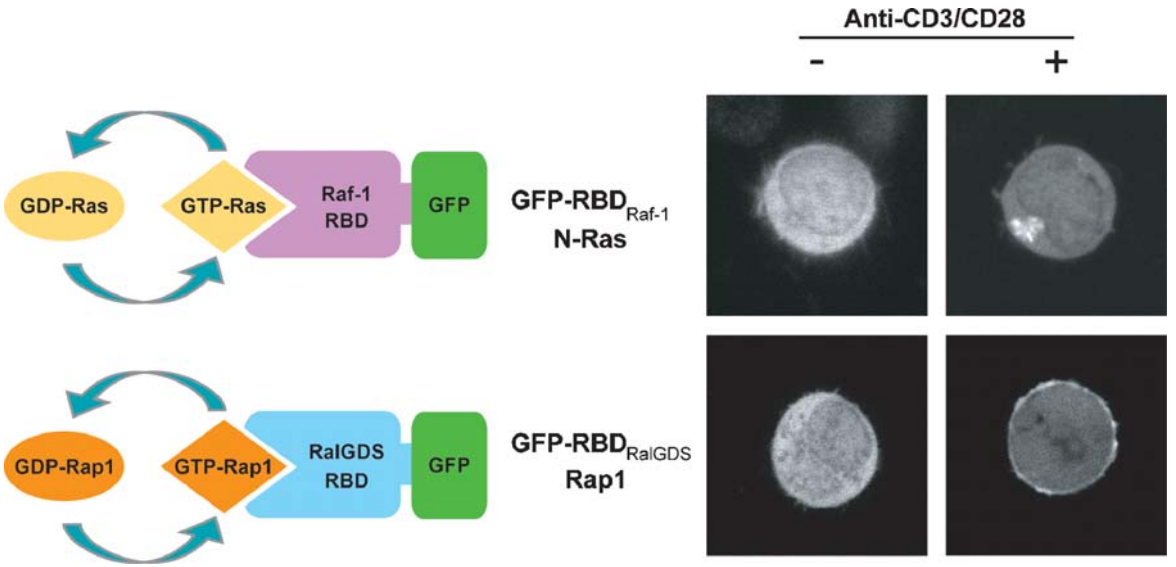

Figure 2

Compartment-specific signaling of Ras and Rap1 in lymphocytes. The Ras-binding domains (RBDs) of various Ras family effectors can be used to construct genetically encoded fluorescent probes of GTPase activity (schematic on *left*) that accumulate upon membranes where the cognate GTPase is activated (confocal micrographs on *right*). The RBD of Raf-1 is specific for active Ras whereas the RBD of RalGDS prefers GTP-bound Rap1. Stimulating Jurkat T cells by cross-linking the TCR results in N-Ras activation on the Golgi but not on the plasma membrane. Conversely, Rap1 is activated only at the plasma membrane.

such as the gut and skin the processes that first encounter antigen can be quite far from the cell body (93). Whether efficient antigen presentation depends on signaling endosomes in these cells remains to be determined.

In addition to providing the most cogent example of long-distance signaling, neuronal cells also provide a well-established model in which the signaling output from endosomes differs from that emanating from the plasma membrane. PC12 cells derived from a rat pheochromocytoma have been used extensively to study neurotropin signaling because the various signaling outputs that include proliferation, cell survival, and differentiation can be separated (94). Whereas short-lived (minutes) MAPK signaling is associated with proliferation, long-term MAPK activity (hours) appears to be required for differentiation (95). Blocking endocytosis with the K44A mutant of dynamin blocked neurite outgrowth (differentiation) in response to NGF but augmented PI3K signaling and cell survival (96). Monodansylcadaverine, a relatively crude inhibitor of endocytosis, blocked Rap1 but not Ras activation in PC12 cells, suggesting a differential requirement for endocytosis in the activation of these related GTPases (97). Interestingly, endocytosis may be differentially required for signaling from the same neurotropin receptor, depending on ligand. Whereas NGF induced internalization of the TrkA receptor and retrograde transport of signaling endosomes, NT-3, which also signals through TrkA, did not (98). Because NGF and NT-3 are required at different stages of neuronal development, this observation represents a dramatic example of compartment-specific signaling at the level of a single receptor. Compartmentalized signaling on endosomes is not restricted to neuronal cells. On gastrointestinal epithelial cells, the PAR2

GPCR signals from β-arrestin-induced endosomes to Erk via a Ras-independent pathway, but a mutant receptor that cannot associate with β-arrestin signals down an alternative, Ras-dependent pathway (99).

Other Systems: TLRs, Smads, and Notch

MAPK pathways are not the only ones that utilize endosome membranes as signaling platforms. Among the other pathways that require endosomes are those involved in antigen processing and innate immunity. For example, Toll-like receptor (TLR) 9 is associated with endosomes that move to the center of the cell upon ligation (100, 101). Whereas signaling of lipopolysaccharide via TLR4 does not require endocytosis, CpG-DNA signaling to TLR9 does (101). TLR signaling from endosomes illustrates another way that compartmentalized signaling on this organelle influences signaling outcome. Plasmacytoid dendritic cells are unique in their ability to mount a robust type I interferon response to TLR9 signaling through MyD88-IRF-7. Whereas ligated TLR9 is relatively rapidly delivered from endosomes to lysosomes in other cell types, the ligand receptor pair lingers for a much longer time in the endosomal compartment of plasmacytoid dendritic cells. Elegant studies designed to alter the endosomal retention time demonstrated that this was the critical variable in mounting an interferon response (102).

TGF-β signaling also uses endosomes. Smad proteins are transcription factors that mediate TGF-β signaling (103). Some Smads, such as Smad2, are tethered to the endosome membrane in association with a protein known as Smad anchor for receptor activation (104). Upon activation, TGF-β receptors internalize into endosomes and phosphorylate Smads (105). Phosphorylated Smads translocate from the endosome to the nucleus to activate transcriptional factor–specific genes.

Notch signaling is a conserved mechanism that transmits signaling between cells that are in direct contact. Signaling is triggered when the Notch receptor at the cell surface binds to ligands of the DSL (Delta, Serrate, Lag2) family, such as Delta, that are presented on the surface of neighboring cells. Ligand binding leads to proteolysis of Notch at two sites, including an intramembranous site cleaved by γ-secretase. Notch cleavage releases an intracellular signaling fragment that regulates gene transcription. Genetic studies in *Drosophila* revealed a critical role for endocytosis in Notch signaling (106). Interestingly, signaling involved endocytosis of the Delta receptor while it was bound to the fragment of Notch from an adjacent cell in a process referred to as trans-endocytosis. New studies reveal that activation of Notch by γ-secretase cleavage requires prior monoubiquitination and endocytosis of the receptor (107).

MAPK Scaffolds

Scaffolding molecules in MAPK pathways were first conceptualized as a means of organizing the various MAPK modules, e.g., Erk, Jnk, and p38 (108). The most dramatic demonstration of this concept was in budding yeast, in which Lim and colleagues (109) were able to rewire the Fus3 (Erk-like) and Hog1 (p38-like) pathways by expressing chimeric versions of the respective scaffolds, Ste5 and Pbs2. An emerging feature of mammalian MAPK scaffolds is that they appear to have specific subcellular localizations and are therefore now recognized as integral to compartmentalized signaling (**Figure 3**). The best-studied of the Erk scaffolds is kinase suppressor of Ras (KSR), first identified in genetic screens in flies (110) and worms (111, 112) as a positive regulator of the Ras/MAPK pathway (KSR is an unfortunate misnomer). KSR is a multidomain protein that binds Raf-1, MEK, and Erk, as well as several other proteins. In resting cells, it is sequestered in the cytosol, like Raf-1, by 14-3-3 proteins. In response to mitogenic signals, KSR becomes dephosphorylated at S392, loses affinity for 14-3-3, and translocates to the plasma membrane (113,

KSR: kinase suppressor of Ras

MP1: MEK partner 1

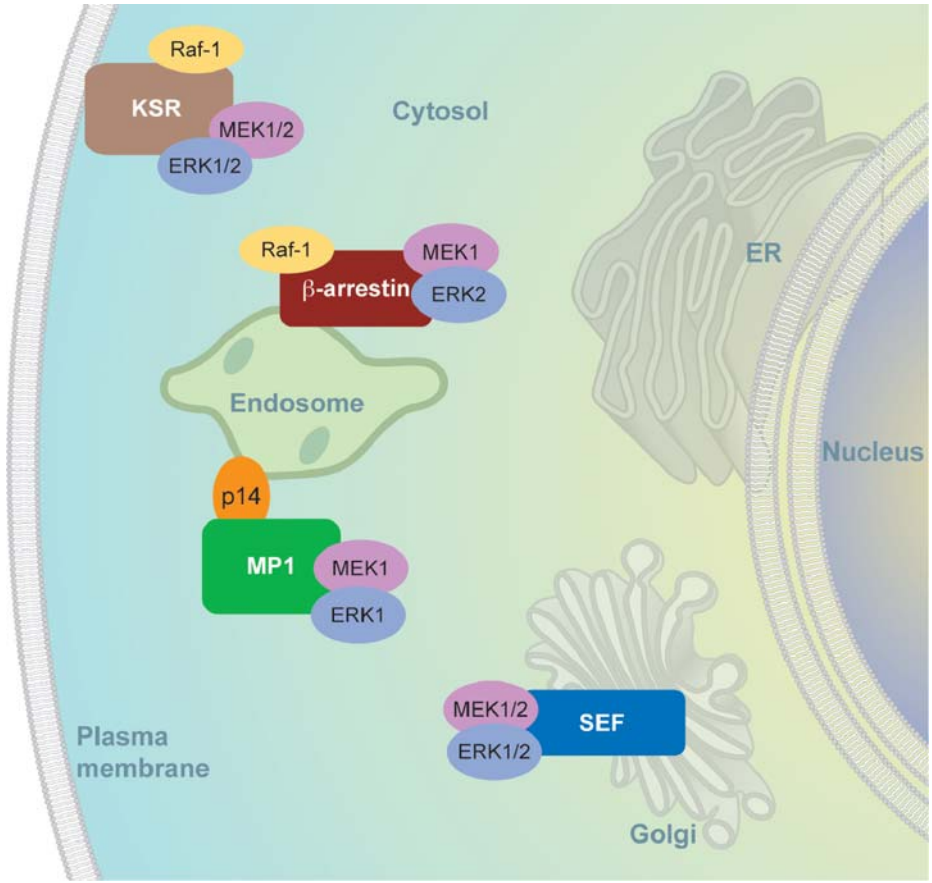

Figure 3

Compartmentalization of MAPK scaffolds. The various MEK/Erk scaffolds have distinct subcellular localizations. Kinase suppressor of Ras (KSR) translocates from the cytosol to the plasma membrane, where it organizes Raf-1/MEK/Erk signaling. MEK partner 1 (MP1) is restricted to early endosomes by p14 and facilitates MEK1/Erk1 signaling on this compartment downstream of growth factor receptors. β-arrestin also localizes to endosomes and serves as a Raf-1/MEK/Erk scaffold downstream of GPCRs. Finally, Sef localizes MEK/Erk complexes to the cytosolic face of the Golgi apparatus and selectively allows phosphorylation of cytosolic substrates such as RSK2.

114) by virtue of a cysteine-rich domain (115). Thus, KSR represents an inducible scaffold for Ras/MAPK signaling with specificity for the plasma membrane. There are at least two *ksr* genes in mammals. Targeted disruption of *ksr1* yielded mice that were relatively normal, although T cell activation was impaired (116).

MEK partner 1 (MP1) was first identified in a yeast two-hybrid screen as a binding partner of MEK1 (117). Further analysis revealed that it bound MEK1 and Erk1 but not MEK2 and Erk2 and that binding facilitated the phosphorylation of Erk1 by MEK1, thus fulfilling the criteria for a MEK/Erk scaffold (117). Interestingly, MP1 was also found to bind p14, a highly conserved protein that resides on the cytoplasmic face of early endosomes (118). Overexpression studies revealed that MP1 could augment Erk signaling but only when coexpressed with p14. Importantly, ectopic targeting of the p14/MP1 complex to the plasma membrane using the C-terminus

of K-Ras failed to augment Erk activation, indicating that the endosomal location is essential for the scaffolding function of p14/MP1. Thus, MP1 is indeed an endosome-specific scaffold for MEK and Erk. By tracking pErk and employing p14 siRNA, Huber and colleagues (118) showed that whereas p14/MP1 was not required for early activation at the plasma membrane, it was required for the activation seen on endosomes 10-30 min after EGF stimulation. This illustrates an important feature of compartmentalized signaling facilitated by localized scaffolds: It allows for the same pathway to be activated in a given cell with different kinetics, thus increasing the complexity of signaling.

MP1 is not the only MAPK scaffold on endosomes; β-arrestin also performs this function in the context of GPCR signaling. Originally characterized as a mediator of GPCR desensitization (119), β-arrestin is now recognized as a multifunctional protein that regulates internalization of GPCRs into CCVs (120, 121) and serves as a scaffold for both the Erk (122) and Jnk (123) MAPK modules. Several lines of evidence implicate β-arrestin functionally in the transmission of signals from Raf-1 to MEK and Erk on endosomes. Raf-1 overexpression increased MEK and Erk binding to β-arrestin (122), and a dominant-negative form of β-arrestin blocked Erk activation downstream of a GPCR (56, 99). Thus, endosomes serve as an organelle that supports both PTKR and GPCR signaling to Erk, and each system uses its own scaffold.

Yet another MEK/Erk scaffold with unique subcellular targeting properties is Sef, a protein originally identified as a negative regulator of fibroblast growth factor signaling (124, 125). Sef was subsequently shown to be a MEK/Erk scaffold that resides on the Golgi apparatus (126). Sef binds only activated MEK. In other systems, Erk dissociates from MEK once it is phosphorylated, and phospho-Erk can then enter the nucleus. However, activated Erk remains associated with MEK on Sef, preventing Erk's entry into the nucleus and sequestering it from its nuclear substrates such as Elk-1. However, the active Erk that remains associated with Sef on the Golgi is capable of phosphorylating cytosolic substrates such as RSK2 (126). When Elk-1 was artificially targeted to the cytoplasm with a nuclear export signal, it became a substrate for Sef-associated phospho-Erk (126). Thus, Sef is a compartment-specific scaffold that steers Erk activity toward one set of substrates over another (127). Another example of shunting toward one set of Erk substrates over another is in GPCR signaling to Erk via β-arrestin, which acts to retain Erk in the cytosol such that cell proliferation is not stimulated (99). Similarly, β-arrestin 2 acts to sequester Jnk3 in the cytosol (123). These examples illustrate another central feature of compartmentalized signaling: The location of the signaling complex can determine which set of downstream effectors is most efficiently activated.

Ras SIGNALING FROM OTHER ENDOMEMBRANES

Signaling on Golgi

Because endosomes derive from the plasma membrane and take with them surface receptors and their ligands, the idea that signaling initiated by extracellular molecules continues on the cytoplasmic surface of these organelles takes no great leap of imagination. This is not the case for other organelles such as the Golgi apparatus, ER, and mitochondria, which are topologically removed from the plasma membrane. Accordingly, until the last few years the membrane platforms for receptor-mediated signaling were thought to be restricted to the plasma membrane and endosomes. The surprising observation that at steady-state a significant pool of N-Ras and H-Ras resides on the Golgi apparatus (20, 128) raised the possibility that Ras/MAPK signaling might also take place on this organelle. This hypothesis was tested by studying Ras activation in living cells using genetically encoded fluorescent probes. The

probe that has been the most informative is relatively simple, consisting of the RBD of Raf-1 fused to GFP (63). This probe reveals Ras activation in a spatiotemporal fashion by recruitment from the cytosol and nucleoplasm to membrane-bound structures. Using this probe Chiu et al. (63) showed that upon stimulation with growth factors Ras became activated at both the plasma membrane and the Golgi. Interestingly, the kinetics of activation were different on the two compartments; activation at the plasma membrane was rapid and transient (1–10 min), whereas activation at the Golgi was delayed (>20 min) and sustained. As discussed above for endosome signaling, this observation suggested that subcellular location of signaling underlies the kinetically distinct peaks of Ras/MAPK signaling that had been observed in the past by biochemical methods (95).

Because retrograde vesicular traffic from the endosomal compartment to the Golgi has been well documented (129), this pathway presented one possibility of how a signal can be delivered from a surface receptor to Ras on the Golgi. However, the signal transmission was too rapid to be explained by vesicular trafficking. Moreover, neither molecular inhibitors of endocytosis nor low temperature affected Ras activation on the Golgi (63). Instead calcium, a diffusible second messenger, and the Ras exchange factor RasGRP1 proved to be responsible for Ras activation on the Golgi (130). RasGRP1 is a member of a family of Ras/Rap1 exchange factors that are activated by diacylglycerol (DAG) and calcium in a fashion analogous to PKCs and other C1 domain–containing proteins (131). Among the four RasGRP proteins, RasGRP1 has the highest degree of specificity for Ras over Rap1 (131) and also proved to have intrinsic affinity for Golgi membranes in activated cells (130, 132–134). Thus, one pathway for Ras activation on the Golgi downstream of PTKRs is via activation of PLCγ, resulting in production of DAG and an increase in intracellular calcium, followed by activation and translocation to the Golgi of RasGRP1, where it acts on Ras (130). This pathway also appears to be dependent on Src, probably at the level of activation of PLCγ (130).

Golgi-associated Ras was initially conceived of as a pool that had not trafficked to the plasma membrane (20). The recent discovery of a plasma membrane/Golgi cycle that functions as a consequence of a palmitoylation/depalmitoylation cycle (35, 36) presents an alternative mechanism for the accrual of active Ras on the Golgi. In this pathway, prenylated but depalmitoylated N-Ras and H-Ras appear to traffic between membrane compartments though the cytosol as fluid phase protein, perhaps bound to a chaperone. In this model, Ras could be activated on one compartment, for example the plasma membrane, and then traffic in a GTP-bound state to another compartment, for example the Golgi. Rocks et al. (36) interrupted the palmitoylation/depalmitoylation cycle by applying the protein palmitoyltransferase inhibitor 2-bromopalmitate and observed a decrease in Ras activation on the Golgi, suggesting that the acylation/deacylation cycle is required for Ras activation on this organelle. Such a model for Ras activation on internal membranes requires an activation-dependent cytosolic pool of GTP-bound Ras that has not as yet been demonstrated. It should be emphasized that a calcium/RasGRP1-dependent and a Ras depalmitoylation–dependent pathway for Ras activation on the Golgi are not mutually exclusive, and both pathways may operate.

As discussed above, very recent work has revealed that the palmitoylated forms of Ras are not the only isoforms that can traffic in a retrograde fashion from the plasma membrane to the Golgi. GFP-tagged K-Ras was seen in rat hippocampal neurons stimulated with glutamate to dissociate from the plasma membrane and associate with intracellular membranes, including Golgi, in a calcium/calmodulin-dependent fashion (37). The physiologic significance of this translocation and whether this is a neuronal-specific process have not been determined.

Signaling on the ER

In addition to evidence of signaling on the Golgi, evidence has been presented for Ras signaling on the ER. When a palmitoylation-deficient form of N-Ras or H-Ras is expressed in cells, it accumulates on the ER and in the cytosol (20). These constructs can be viewed as surrogates either for the biosynthetic intermediates in the trafficking pathway of nascent Ras or for mature Ras that has reached the plasma membrane and then become depalmitoylated. When this construct was expressed along with the GFP-RBD spatiotemporal reporter of Ras activation, GTP-bound Ras accumulated on the ER following growth factor stimulation in less than one minute (63). One interpretation for this result is that GEFs such as RasGRP1 can rapidly activate ER-associated Ras in situ. Evidence to support this idea has been presented by Arozarena et al. (134a) who showed that Ras guanine nucleotide-releasing factors (RasGRFs), calcium-regulated Ras GEFs that are highly expressed in the neurons, translocate to the ER and activate Ras on this compartment. Given the recent evidence for the highly dynamic nature of the membrane binding of Ras proteins that are modified only with a prenyl group (35, 36), it seems plausible that under these conditions Ras becomes active on another compartment such as the plasma membrane. Whereas the steady-state localization of palmitoylated Ras proteins clearly includes the Golgi, GFP-Ras is only observed on the ER if palmitoylation is blocked (20). This observation calls into question the physiologic significance of signaling from the ER.

Perhaps the strongest evidence for Ras signaling from the ER comes from the discovery of a Ras effector that is restricted to that compartment. Sobering et al. (135) described a protein in *Saccharomyces cerevisiae* designated ER-associated Ras inhibitor (Eri1) that behaved genetically like an inhibitor of Ras, bound preferentially to GTP-bound Ras via its effector domain, and was localized to the ER. Further analysis revealed Eri1 was an integral component of GPI (glycosylphosphatidylinositol)-GlcNAc transferase, an ER-restricted enzyme that catalyzes the transfer of UDP-GlcNAc to an acceptor phosphatidylinositol, the first and rate-limiting step in the biosynthesis of GPI anchors. Interestingly, GTP-bound Ras acted to inhibit the enzyme, making GPI-GlcNAc transferase the first Ras effector that is negatively regulated by active Ras (136). The physiologic significance of the regulation of GPI anchor synthesis by Ras is not clear, but given the critical roles of GPI-anchored proteins in many systems, including the immune system, and given the fact that somatic mutations in a component of GPI-GlcNAc transferase in hematopoetic stem cells leads to a human disease known as paroxysmal nocturnal hemaglobinuria, it is an area of Ras biology that will attract considerable attention.

Signaling in or on Mitochondria

Mitochondria have long been neglected as potential sites of signal transduction. The recognition over the past decade of this organelle as the master gate keeper of programmed cell death has caused cell biologists to be more open-minded regarding signaling and mitochondria. Several signaling molecules have been found in mitochondria. For example, c-Src was found to regulate cytochrome c oxidase in the mitochondria of osteoclasts (137). Recently, EGFR was found to translocate to mitochondria and associate with subunit II of cytochrome c oxidase via pY845, which is a Src phosphorylation site (138). In addition to these tyrosine kinases, a tyrosine phosphatase has been described that is targeted to the inner mitochondrial membrane (139).

Components of the Ras/MAPK pathway have been reported on mitochondria. Using subcellular fractionation, Rebollo et al. (140) reported all three Ras isoforms in mitochondrial fractions of an IL-2-dependent murine T cell line and showed that the Ras proteins could be coprecipitated with Bcl-2

from these fractions. Interestingly, whereas the association of K-Ras with mitochondria was IL-2 dependent, H-Ras association was only seen after IL-2 withdrawal. Surprisingly, mitochondrial association was not dependent on CAAX processing, calling into question the biological relevance of the observation. Moreover, these results are at odds with the extensive literature on Ras localization by microscopy in which mitochondrial localization has not been reported. The discrepancy may relate to the nonspecific stickiness of Ras proteins for membrane fractions ex vivo. Nevertheless, the idea of Ras/MAPK signaling from mitochondria is intriguing. Wang et al. (141) showed that Raf-1 could be recruited to mitochondria by Bcl-2, and from that compartment it could phosphorylate and inactivate the proapoptotic protein BAD. In these studies, evidence for the association of endogenous Raf-1 with mitochondria was lacking, and, as for Ras, an extensive literature on Raf-1 localization by microscopy does not contain evidence of mitochondrial association. Recently, we have shown that K-Ras phosphorylated by PKC in its polybasic region loses affinity for the plasma membrane and translocates to intracellular membranes, including the outer mitochondrial membrane. Using immunogold electron microscopy, we observed PKC-dependent translocation of endogenous Ras to mitochondria in Jurkat T cells. Interestingly, phospho-K-Ras promoted apoptosis in a Bcl-XL-dependent fashion (141a).

Role of Compartment-Specific Signals

What is the physiologic significance of Ras signaling from intracellular membranes? As discussed above in the context of endosomes, compartmentalized signaling, in theory, can increase the complexity of signaling by adding kinetically distinct outputs down a single pathway and/or by allowing for activation of distinct downstream pathways. This increase in complexity may help explain how a single regulatory molecule such as Ras can control such a plethora of cellular responses. Evidence in support of the compartmentalized signaling model has come from experiments in which transmembrane tethers were used to artificially and stringently target Ras proteins to various membrane compartments (63). When oncogenic Ras was targeted to the ER or Golgi with a transmembrane tether, it retained full transforming activity, indicating that all the signaling events required for the complex cellular phenotype of transformation can be set into motion from internal membranes (63, 133). This might suggest that Ras signaling from internal membranes is no different than from the plasma membrane. However, quantitative differences in signal output could be detected. Whereas Golgi-associated Ras activated Erk and PI3K with a potency equal to that of natively targeted Ras, the Jnk pathway was poorly activated. Conversely, ER-tethered Ras was a potent activator of Jnk but a relatively poor activator of Erk and PI3K (63). The most compelling evidence for compartmentalized Ras signaling has come from the study of fission yeast. In *Schizosaccharomyces pombe*, Ras1 controls both the mating pathway via a MAPK cascade and elongated cellular morphology through an exchange factor for Cdc42 (142). ER-restricted Ras1 can support morphology but not mating and the converse is true for Ras1 restricted to the plasma membrane (B. Onken, M.R. Philips & E.C. Chang, manuscript submitted).

PLASMA MEMBRANE MICRODOMAINS AND Ras SIGNALING

As discussed above, the concept of compartmentalized signaling relates to different organelles. However, compartmentalized signaling can also apply to subcompartments within a given organelle. The best-studied example of this relates to the plasma membrane, where microdomains and their relationship to signaling events have been

extensively studied for a decade. The plasma membrane microdomain that has received by far the most attention is the lipid raft (143). Lipid rafts are microdomains rich in cholesterol and sphingolipids that are thought to maintain a liquid-ordered state and thereby partition into patches within the disordered glycerophospholipids of the bulk membrane. In cells that express caveolins, one type of lipid raft forms structures known as caveolae that are readily observed by electron microscopy (144).

Glycophosphatidylinositol-anchored proteins partition into the outer leaflet of lipid rafts, whereas some lipidated cytosolic proteins such as Src family kinases partition into the cytosolic leaflet. The observation that many of the proteins that partition into lipid rafts are involved in signaling has led to the hypothesis that lipid rafts are hot spots for signal transduction (145). Because lipid rafts have been observed in living cells only with indirect methods, such as resistance to detergent extraction, their biological significance has yet to be proven (146).

The association of components of the Ras/MAPK pathway with lipid rafts is somewhat controversial because the list depends on the methods used to isolate the microdomains. Among the Ras/MAPK signaling components that have been associated with lipid rafts are growth factor receptors, including EGFR, PDGFR, and the insulin receptor. Interestingly, EGFR partitions in and out of caveolae in a ligand-dependent fashion (147). The biophysical basis for the partitioning into lipid rafts of a transmembrane protein lacking lipid modification such as PTKRs is not understood. In contrast, the partitioning of acylated proteins such as Src family kinases into lipid rafts can be understood as a function of the ability of acyl chains to insert into phospholipid bilayers (148). Unlike acylated proteins, prenylated proteins are generally excluded from detergent-resistant membrane fractions (149). Nevertheless, Ras proteins have been reported in caveolin-enriched fractions made without detergent (150). For N-Ras and H-Ras that are both acylated and prenylated, the acyl chain is apparently dominant and can cause these Ras isoforms to partition into lipid rafts. Ras also interacts directly with caveolin-1 (151), providing an additional mechanism for recruitment to these specialized membrane microdomains.

With regard to the role of lipid microdomains in Ras signaling, the most compelling analysis has come from the work of Hancock and Parton and their colleagues. Roy et al. (69) showed that a dominant-negative form of caveolin inhibited H-Ras but not K-Ras signaling and that this differential effect could be mimicked by cholesterol depletion. These data represent the first demonstration that Ras isoforms operate in functionally distinct plasma membrane microdomains. Using multiple methods, including detergent-free subcellular fractionation, immunogold electron microscopy, and immunofluorescence, Prior et al. (152) showed that H-Ras was associated with lipid rafts, whereas K-Ras was not. Moreover, access of H-Ras to lipid rafts was dynamic; GDP-bound H-Ras was favored, suggesting that activation takes place in rafts, but effector engagement occurs in nonraft domains (152). These investigators went on to develop an elegant method for direct visualization of membrane protein clustering using immunogold electron microscopy staining of exposed membrane sheets, followed by statistical point pattern analysis (153). These studies confirmed that whereas GDP-bound H-Ras clustered in domains with a mean diameter of 44 nm that were sensitive to cholesterol depletion, GTP-bound H-Ras and all forms of K-Ras clustered in domains that were insensitive to cholesterol depletion (153). Combining this new method with FRAP (fluorescence recovery after photobleaching) studies and more extensive mutational analysis revealed that the partitioning of H-Ras into lipid rafts is governed by multiple factors that include the acyl chains, the adjacent hypervariable region of the C-terminus, and the GTP-binding domain (154).

Microdomain studies have recently been complemented with single-particle tracking studies of Ras. Murakoshi et al. (155) have tracked YFP-tagged Ras at the level of single molecules and observed random motion in the plane of the membrane interspersed with periods of immobility that last <1 s. Using FRET between YFP-Ras and Bodipy TR-GTP, these investigators have shown that the periods of immobility correspond to GTP loading. Growth factor stimulation increased the proportion of Ras molecules that are immobilized. Interestingly, these investigators did not observe isoform differences in these behaviors (155). These observations have been interpreted to suggest that nanoclusters of activated Ras and other components of the Ras signaling pathway exist. The mechanism of the nanoclusters' formation may relate to a corral-like effect imposed on active Ras by cytoskeletal elements, combined with transmembrane proteins that act as pickets (156) or membrane microdomains containing disordered lipids (157). Either way, the plasma membrane clearly can no longer be considered a homogeneous platform for cellular signaling. Similar to organelle-specific signaling, the heterogeneity of plasma membrane microdomains may contribute to the complexity of signaling outputs.

COMPARTMENTALIZED SIGNALING IN LYMPHOCYTES

T Cell Signaling and Lipid Rafts

Much of the evidence for lipid rafts serving as signaling platforms comes from the study of T lymphocytes (158, 159). TCR and associated signaling molecules were enriched in detergent-resistant microdomains following TCR activation, and TCR signaling could be inhibiting by disrupting lipid rafts (160). Magee and colleagues (161) developed a method for visualizing, at the resolution of the light microscope, lipid rafts on the surface of lymphocytes by capping a GPI-anchored protein using the cholera toxin B subunit (CT-B). When rafts were capped, so too were TCR, Lck, and LAT (linker-activated T cell). Conversely, activating the TCR by capping with cross-linking antibodies also patched CT-B, Lck, and LAT (161). The earliest events in TCR signaling involve the Src family kinases Lyn and Fyn that are doubly acylated, explaining their affinity for lipid rafts. Interestingly, LAT, a transmembrane protein that serves as a scaffold for the phosphotyrosine docking sites of several signaling molecules (162), is palmitoylated, and this modification likely explains its constitutive affinity for lipid rafts (163). Co-stimulation from CD28 has also been explained in the context of lipid rafts (164). Some accessory molecules in TCR activation, such as CD48, are GPI-linked, and their partition into lipid rafts has been implicated in augmenting TCR signaling (165). Several lines of evidence have implicated lipid rafts in the formation of the immunological synapse (158, 159). However a very recent study that used single-molecule tracking showed that rafts were not required for the clustering of CD2, Lck, and LAT, calling into question the role of lipid microdomains in immunological-synapse formation and TCR signaling (166).

Compartmentalized Ras Signaling in T Cells

Although all three Ras isoforms are expressed in T lymphocytes, the levels of H-Ras are much lower than those of N-Ras and K-Ras. There are several reasons to conclude that N-Ras has particular significance in T cell signaling. First, in contrast to carcinomas where *kras* mutations predominate, when lymphoid malignancies are associated with oncogenic *Ras* mutations (30% of cases) they are almost always in the *nras* gene (10). Second, mice deficient in N-Ras have a defect in T cell function and are extremely sensitive to infection with influenza virus (167). Finally, although K-Ras can be activated by robust TCR signaling, under conditions of low-level

cross-linking of TCR with anti-CD3 antibodies, N-Ras is preferentially activated (132).

Surprisingly, when TCRs were capped on Jurkat T cells with anti-CD3 antibodies such that lipid raft clusters were formed as indicated by cocapping of CT-B, GFP-H-Ras was recruited to the clusters but GFP-N-Ras was not (132). Less surprising was the absence of GFP-K-Ras from the clusters, because this isoform lacks an acyl chain. Interestingly, constitutively active GFP-H-Ras12V was excluded from the clusters (132), consistent with the findings of Prior et al. (152) indicating that activated H-Ras moves out of lipid rafts. Thus, if the hypothesis that N-Ras signaling is critical to T cell function is correct, this Ras isoform does not appear to colocalize with the receptor under conditions of T cell signaling.

Another surprise came when GFP-RBD, the in vivo probe for GTP-bound Ras described above, was applied to Jurkat T cells. Concordant with the earlier studies of fibroblasts, N-Ras and H-Ras were activated on the Golgi apparatus following TCR activation. However, this was the only membrane compartment upon which GTP-Ras could be detected; the plasma membrane was entirely devoid of activity. Unlike fibroblasts, where activation of Ras on the Golgi was delayed, activated Ras could be detected on the Golgi within minutes of stimulation. RasGRP1 is much more highly expressed in lymphocytes than in fibroblasts (168), and the difference in kinetics of Ras activation on the Golgi may simply be a reflection of this difference. Recently, these observations in Jurkat T cells were extended to primary murine T cells where GFP-RBD reports Ras activation only on the Golgi apparatus following TCR activation by cross-linking CD3 and CD28 (**Figure 4**) (A. Mor & M.R. Philips, unpublished observation). In addition, the GFP-RBD probe revealed constitutive Ras activation in Raji B cells that occurred only on the Golgi suggesting that, as in T cells, Ras signaling in B cells occurs on the Golgi (A. Mor & M.R. Philips, unpublished observation).

Endogenous N-Ras was localized to the Golgi of Jurkat T cells (132), and YFP-RasGRP1 translocated to this organelle in response to TCR signaling or stimulation with phorbol myristate acetate and ionomycin (130, 132). Unlike RasGRP2 and RasGRP4, both RasGRP1 and RasGRP3 have affinity for the Golgi in Jurkat T cells. However, overexpression only of RasGRP1 leads to Ras activation on the Golgi (132). A dominant interfering mutant of RasGRP1 inhibited activation on this compartment (130). Silencing of the *RasGRP1* gene with siRNA abolished all Ras signaling (i.e., recruitment of GFP-RBD to the Golgi), demonstrating that this exchange factor is responsible for activating Ras on the Golgi of T cells (130). This result is concordant with studies of RasGRP1-deficient mice in which Ras signaling, as determined biochemically, was eliminated (16). Also consistent with RasGRP1 being responsible for the activation of N-Ras on the Golgi of Jurkat T cells was the absence of any activation observed in T cells deficient in $PLC_{\gamma 1}$ (130, 132). Low concentrations of anti-CD3 antibodies induced N-Ras activation on the Golgi but did not activate K-Ras. However, high concentrations of anti-CD3 antibody activated K-Ras, and the GFP-RBD probe indicated that this activation occurred on the plasma membrane (132). Thus, K-Ras can be activated on the plasma membrane, presumably via signaling through Grb2/SOS, provided that the stimulus is strong (132).

N-Ras is expressed on both the plasma membrane and Golgi of Jurkat T cells. What accounts for activation on only one of these compartments when exchange factors are present on both? One explanation is that there may be a Ras GAP that inactivates Ras specifically at the plasma membrane. CAPRI is a Ras GAP that operates at the plasma membrane and is an attractive candidate for a component of Ras signaling in lymphocytes because it is calcium activated (169). Knockdown of CAPRI in Jurkat T cells with siRNA resulted in H-Ras activation on the plasma membrane following TCR stimulation (130).

CAPRI: calcium-promoted Ras inactivator

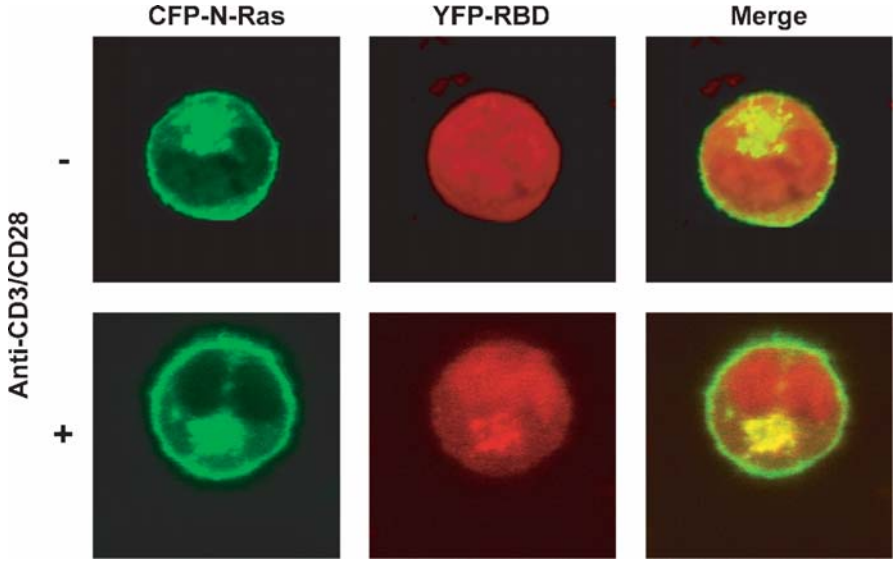

Figure 4

Ras activation only on the Golgi of primary T cells. $CD4^+$ splenocytes from Balb/C mice were transfected with CFP-N-Ras and YFP-RBD. N-Ras is expressed in resting cells on both the plasma membrane and Golgi. Following cross-linking of the TCR with anti-CD3/CD28 antibodies, YFP-RBD rapidly (<5 min) accumulated on the Golgi apparatus but not on the plasma membrane.

Thus, CAPRI appears to keep Ras signaling on the plasma membrane of lymphocytes in check while signaling proceeds on the Golgi. Interestingly, because both CAPRI and RasGRP1 are activated by calcium, this single second messenger controls Ras signaling in opposite directions simultaneously on different subcellular compartments (**Figure 5**), a stark example of compartmentalized signaling. The physiological reason why Ras signaling in T cells occurs primarily on the Golgi remains to be elucidated.

CONCLUSION

The evidence for Ras/MAPK signaling on intracellular membranes is strong. The association of PTKRs and downstream elements of the Ras/MAPK pathway with endosomes was first demonstrated by subcellular fractionation and conformed to the paradigm of desensitization through endocytosis. Functional studies using molecular inhibitors of endocytosis revealed that endocytosis had to be more than a mechanism to limit receptor signaling because in many cellular contexts it was required for signal propagation. GFP-tagged probes confirmed that active Ras/MAPK signaling occurs on the cytosolic surface of endosomes. These probes also revealed signaling on other endomembranes, including the Golgi apparatus. The characterization of various MAPK scaffolds that are restricted to different membrane compartments, including the plasma membrane, endosomes, and Golgi, further explained the mechanisms of compartmentalized signaling.

With regard to Ras activation on internal membranes, a spatiotemporal fluorescent probe has revealed that the pathway of activation on the Golgi involves PLCγ and RasGRP1 and thereby differs from the Grb2/SOS-mediated pathway for Ras activation on the plasma membrane. Thus, compartmentalized signaling can be accomplished by using distinct upstream pathways. The

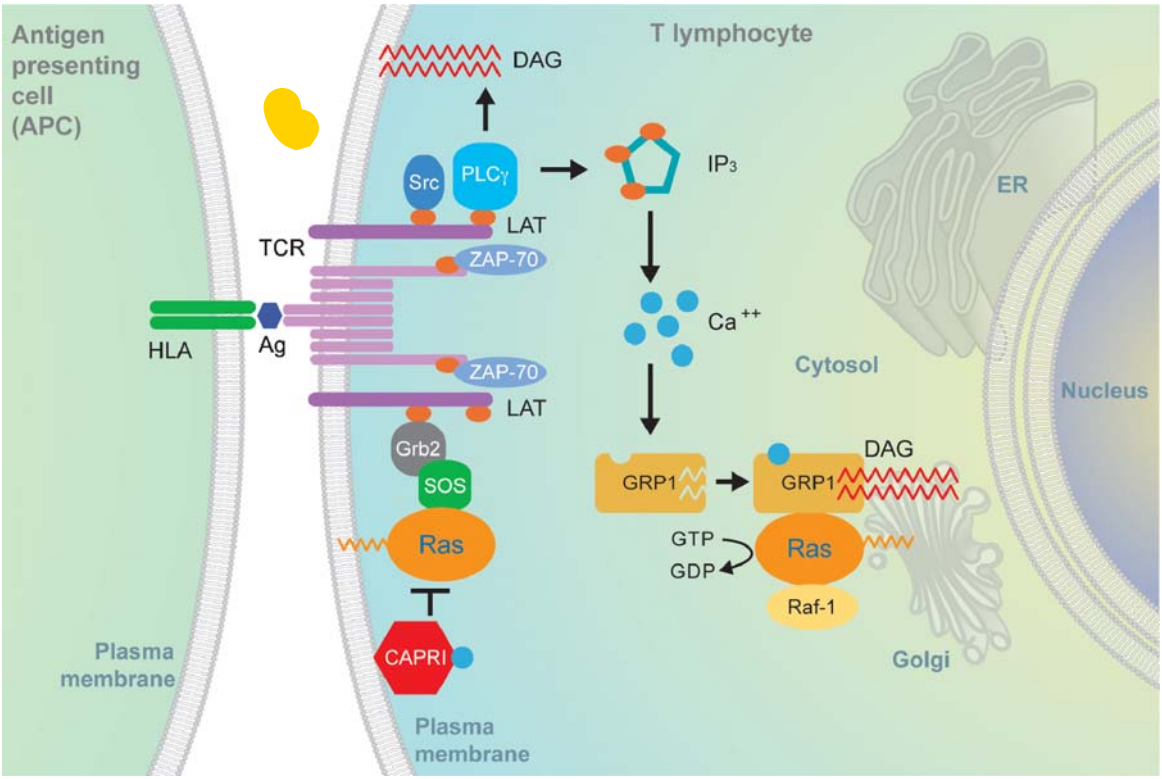

Figure 5

PLCγ and RasGRP1 mediate Ras activation on the Golgi of T lymphocytes, and CAPRI limits activation at the plasma membrane. Activation of the TCR results in tyrosine phosphorylation of the ζ chain of the receptor by Src family kinases and the resulting phosphotyrosines serve to recruit ZAP-70, which in turn phosphorylates the scaffold protein LAT at multiple sites. Among the signaling molecules recruited to phosphorylated LAT is PLCγ, which acts on phosphatidylinositol-4,5-bisphosphate in the plasma membrane to produce diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). Calcium liberated from internal stores by IP_3 acts on the calcium- and DAG-sensitive Ras exchange factor RasGRP1 and causes it to translocate to the Golgi, where DAG levels are relatively high. RasGRP1 activates Golgi-associated Ras on this compartment. Meanwhile, calcium also activates the Ras GAP CAPRI that translocates to the plasma membrane and downregulates any Ras that is activated on this compartment by the exchange factor SOS.

PLCγ/RasGRP1 pathway appears to be dominant in T lymphocytes in which all Ras signaling downstream of the TCR was observed on the Golgi apparatus.

More difficult than establishing Ras/MAPK signaling on endomembranes will be understanding the physiologic significance of compartment-specific signaling. The most obvious purpose of compartmentalized signaling is to increase the complexity of signal output by extending and segregating the signaling repertoire of critical regulatory molecules such as Ras. However, evidence for differential signal outputs from different compartments is sparse and more or less confined to overexpression studies of signaling molecules that are rerouted to ectopic compartments. Elucidation of the spatial complexity of signaling networks in a physiologic context represents one of the next frontiers in signal transduction research.

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126. This study showed that Sef is a Golgi-localized MAPK scaffold that activates MEK/ERK complexes and permits phosphorylation of cytosolic but not nuclear substrates.

130. GFP-RBD was used to map the signaling pathway for in situ activation of Ras on the Golgi, which involves Src, PLCγ, calcium, and RasGRP1.

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