Agonist-dependent Traffic of Raft-associated Ras and Raf-1 Is Required for Activation of the Mitogen-activated Protein Kinase Cascade*

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Stimulation of HIRcB fibroblasts with insulin leads to accumulation of active components of the mitogen-activated protein kinase cascade in endocytic compartments. However, the factors that regulate the mobilization of these components through the endocytic pathway and the relevance of this event to cellular signaling remain unclear. Here we report that Ras proteins are associated with lipid rafts in resting HIRcB fibroblasts. Ras is rapidly internalized into the endocytic compartment following stimulation with insulin. The redistribution of Ras is independent of its activation. Attachment of the C-terminal 20 amino acids of Ha-Ras to green fluorescent protein was sufficient to target this construct to the same loci as the endogenous Ras protein, indicating that Ras distribution is a consequence of the association of its lipid modified C terminus with membranes. Depletion of plasma membrane cholesterol localized Ras and blocked insulin-dependent Ras trafficking. Cholesterol depletion also blocked insulin-dependent phosphorylation of MEK and mitogen-activated protein kinase (MAPK) but had no effects on the translocation and activation of Raf-1. A second inhibitor of endocytosis, cytochalasin D, also blocked insulin-dependent MAPK phosphorylation. Taken together, these results suggest that mobilization of active Raf-1 through the endocytic compartment is required for completion of the MAPK cascade.

Stimulation of growth factor receptors leads to the activation of the mitogen-activated protein kinase (MAPK)* cascade. Central to this process is the activation of the small GTPase Ras (1), which, upon binding GTP, associates with the serine/threonine kinase Raf-1. Ras-Raf-1 association results in the activation of the extracellular signal-regulated kinase family of MAPKs. Genetic models have provided exceptional evidence that supports this view (2). However, a number of events must occur to get efficient transduction of the signal. In a resting cell, the components of the MAPK cascade exist in different subcellular compartments. Raf-1 must translocate to the plasma membrane (3, 4) to interact with membrane-bound Ras proteins. This translocation is regulated by its association with phosphatidic acid. Disruption of the interaction between Raf-1 and phosphatidic acid, either by mutation of Raf (5) or by blocking the activation of phospholipase (6), prevents agonist-dependent activation of the MAPK cascade. In general, most current models of signal transduction propose the formation of multimeric complexes at specified loci as critical steps in the regulation of signaling pathways.

Acidic lipid second messengers, such as polyphosphoinositides (7) and phosphatidic acid (6), are important regulators of protein compartmentalization. Many proteins, including EEA-1 (8), protein kinase B (9), and Raf-1 (5, 6, 10), require association with acidic phospholipids for proper targeting. In addition to their role in protein recruitment, acidic phospholipids also possess distinct biophysical properties that are relevant to cellular signaling. In the presence of divalent cations, such as calcium, many acidic phospholipids cluster together and phase separate in model membrane systems. Not surprisingly, at least some of these lipids have been found in cellular lipid domains that contain similar biophysical characteristics (11). These domains, which include lipid rafts, are characterized by phase separation, resistance to solubilization in non-ionic detergents, and cholesterol enrichment.

The biological functions of lipid rafts have not been well defined. However, the metabolism of acidic phospholipids and the maintenance of the structure of lipid rafts have been found to be crucial to vesicular traffic from the plasma membrane. Depletion of plasma membrane cholesterol disrupts clathrin-mediated endocytosis (12, 13), and enzymes that generate acidic phospholipids play a critical role in vesicle fission (14). Furthermore, the generation of phosphatidylinositol phosphates and phosphatidic acid promotes internalization of vesicles from the plasma membrane (15). Both lipid raft structures and acidic phospholipids are clearly important elements in vesicle transport from the plasma membrane.

There is a substantial body of evidence linking endocytosis to the regulation of signaling events. In fact, it has been proposed that some of these, such as the Ras-MAPK cascade, might occur on endocytic vesicles. For instance, Pol et al. (16) have reported that the components of the MAPK are associated with endosomes in epidermal growth factor-treated rat livers. Previous
work from our laboratory has shown similar results in insulin-
stimulated HIRcB cells (5, 6). Therefore, the components of the
MAPK cascade appear to accumulate on the surface of endo-
somes. However, the mechanisms through which these pro-
teins associate to endocytic vesicles and the functional rele-
ance of the redistribution of these signaling molecules are not
well understood.

Therefore, we examined the redistribution of Ras from the
plasma membrane to the endocytic compartment. Endogenous
Ras in HIRcB fibroblasts colocalized with cholesterol and glyco-
sphosphatidyl inositol (GPI)-anchored proteins, two markers
for lipid rafts. After stimulation with insulin, Ras was found
associated with immunopurified vesicles that were resistant
to extraction with Triton X-100, suggesting that agonist-dependent
Ras traffic occurs through a subset of cholesterol-rich endosomes,
probably derived from lipid rafts. Furthermore, green fluorescent
protein tagged Ha-Ras (GFP-Ras) was also localized in lipid rafts
and was mobilized through the endocytic pathway in re-
sponse to insulin. Agonist-dependent GFP-Ras traffic was inde-
pendent of its association with GTP, and a 20-amino acid peptide
containing the farnesylation and acylation motifs of Ha-Ras was
sufficient to target GFP to the correct subcellular localization.
Depletion of cellular cholesterol by cyclodextrin treatment dis-
rupted traffic of GFP-Ras. Furthermore, cyclodextrin treatment
also inhibited insulin-dependent MEK and MAPK phosphoryla-
tion without affecting Raf-1 activation. Cytochalasin D also
inhibited Ras internalization and MAPK phosphorylation. These
results indicate that translocation of the components of the
MAPK to the surface of endocytic vesicles is required for activa-
tion of MEK by Raf-1.

EXPERIMENTAL PROCEDURES

Materials and Constructs—Ha-Ras and the Ha-Ras CAAX motif (C-
terminal 20 amino acids) were cloned into pEGFP-C1 (CLONTECH).
Anti-Ras antibodies (clone Ras10) were obtained from Upstate Biotech-
nologies. Mouse anti-c-Raf-1 antibodies were obtained from Transduc-
tion Laboratories. Phospho-specific MEK and MAPK antibodies
were obtained from New England Biolabs, and anti-GFP antibodies
were from CLONTECH. All other materials were purchased from Sigma
unless otherwise noted. Anti-GPI antibodies were prepared as de-
scribed previously (17).

Cell Culture—Rat-1 fibroblasts that overexpress insulin recep-
tors (HIRcB cells) were cultured in Dulbecco’s modified Eagle’s medium/
Ham’s F-12 medium (Life Technologies, Inc.) supplemented with 10%
fetal bovine serum (Life Technologies, Inc.) as described previously (6).
Superfect transfection reagent (Qiagen) was used to introduce plasmid
DNA into HIRcB cells for imaging studies, whereas LipofectAMINE
(Life Technologies, Inc.) was used for biochemical studies. Transfection
efficiencies greater than 70% were achieved using LipofectAMINE as
determined by fluorescence and differential interference contrast micro-
scopy of the transfected cells.

Fluorescence Microscopy—Cells grown on poly-l-lysine coated cover-
slips (6) were fixed in 3% paraformaldehyde/phosphate-buffered saline
(30 min, 4 °C) prior to permeabilization with 0.1% Triton X-100 (2 min)
or filipin (150 µg/ml in 3% bovine serum albumin). Permeabilized cells
were stained with anti-Ras (1:500 in 3% bovine serum albumin, phos-
hate-buffered saline) followed by Cy5-conjugated secondary (Jackson
Immunoresearch) or with anti-Ras and rabbit anti-GPI antibodies with
fluorescein isothiocyanate- and Cy5-conjugated secondary. Observa-
tion of fixed specimens was with a Leica-TCS confocal microscope using
filters and laser lines appropriate for fluorescein isothiocyanate and
Cy5 or with an Olympus Provis equipped with an Optronics “Mag-
nafire” camera with filters appropriate for filipin, fluorescein isothio-
cyanate, and Cy5.

Live cell epifluorescence images were acquired on a Nikon Diaphot
inverted microscope equipped with a cooled CCD camera (Photomet-
Instrumente) and appropriate excitation and emission filters for GFP.
Images were captured and processed using Inovision ISEE software.
Experiments on this system were performed at 37 °C as described (6).
Evanescent wave microscopy was performed as described by Han et al.
(18). Total cellular fluorescence was quantitated using Inovision ISEE
software. Background fluorescence was subtracted from each image.
Each image was then normalized to the total cellular fluorescence prior
to cellular stimulation (100%). The images were processed and prepared
using Adobe Photoshop 5.0 software.

Immunosoliation of Vesicles—Endocytic vesicles were purified from
HIRcB cells as previously (6). Prior to immunosoliation, purified ves-
icles from six dishes were split into three equal fractions. Immunos-
oliation of endocytic vesicles was performed as described previously (6)
in the absence of detergent, with 1% Triton X-100, or 1% cholate.
Immunosolates were then analyzed by Western blot as described pre-
viously (6).

Cholesterol Depletion—Cells were treated with 2% β-methyl cyclo-
dextrin (CD)/Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium
for 30 min and washed three times with phosphate-buffered saline to
deplete plasma membrane cholesterol. CD treatment reduced total
cholesterol content from 62.69 ± 8.64 to 11.97 ± 2.72 ng/µg protein as
determined by the Amplex Red Cholesterol Oxidase Assay (Molecular
Probes). Cholesterol-CD inclusion complexes were prepared as de-
scribed by Klein et al. (19).

RESULTS

We have previously reported that insulin stimulates the
redistribution of Ras to early endosomes in Rat-1 fibroblasts
that overexpress the human insulin receptor (HIRcB cells) (5).
However, the dynamics of this process and the mechanisms
involved in targeting Ras to endosomes have not been de-
scribed. Cholesterol-rich microdomains known as lipid rafts
have been shown to contain significant levels of Ras (20) and to
play a role in the regulation of endocytic processes (12, 13, 21).
Therefore, the specific association of Ras with cholesterol-rich
structures may be relevant to the mechanisms that regulate
intracellular Ras traffic. We therefore examined the distribu-
tion of Ras on the plasma membrane of HIRcB fibroblasts and
compared it with the distribution of two known components of
lipid rafts, cholesterol, and GPI-anchored proteins (Fig. 1A).
GPI-anchored proteins were clearly localized on discrete re-
gions of the plasma membrane, as described by others (22).
These regions were stained with filipin and anti-Ras antibo-
dies, indicating that these structures also contained cholesterol
and Ras. However, cholesterol and Ras staining was not lim-
ited to regions containing GPI-anchored proteins (Fig. 1A, ar-
rows 2). These results suggest that only a pool of Ras is asso-
ciated with lipid rafts.
Characterization of Agonist-dependent Ras Traffic

We have observed Ras redistribution to endosomes shortly after the stimulation of cells with insulin. To further examine these endosomes, endocytic vesicles were purified by sucrose gradient followed by immunosialation using antibodies specific to the insulin receptor. Vesicles containing Ras, Raf-1 and phosphorylated MAPK were resistant to extraction with Triton X-100 (Fig. 1C), suggesting that these vesicles were highly enriched in cholesterol. Furthermore, these vesicles were soluble in cholate, a bile salt detergent that efficiently solubilizes cholesterol-rich membranes.

These data suggested the hypothesis that a subpopulation of Ras primarily associated to cholesterol-rich microdomains traffics while attached to the cytoplasmic surface of endosomes in response to insulin. To further characterize this phenomenon, we examined the dynamics of a green fluorescent protein-Ras fusion protein (GFP-Ras) in resting and insulin-treated live cells. Like the endogenous protein, GFP-Ras fluorescence and filipin staining were colocalized in punctate regions of the plasma membrane prior to agonist stimulation (Fig. 2A). After insulin stimulation, a significant fraction of GFP-Ras was also found associated to endocytic vesicles that were positively stained with filipin (Fig. 2A).

Several interesting observations arise from the examination of the dynamics of agonist-dependent GFP-Ras traffic in live cells. Prior to stimulation, GFP-Ras was associated to the plasma membrane and to a perinuclear region that has been previously identified as the Golgi apparatus (23). Following insulin stimulation, plasma membrane-bound GFP-Ras was rapidly internalized. Interestingly, GFP-Ras internalization was most conspicuous on the leading edge of the fibroblast. This is similar to the findings of Chen et al. (24), who found that the Ras activator Sos was preferentially recruited to the leading edge of fibroblasts, although the causes of this apparent polarization are unclear. A GFP-Ras mutant that is locked in the inactive conformation was also found to traffic like the wild-type protein, indicating that regulation of Ras localization is independent of its state of activation. These observations suggested that the specific association of Ras to cholesterol-rich microdomains might be determined by the interactions of the lipid-modified C terminus of Ras with the lipid membrane. To test this hypothesis, a fragment containing the C-terminal 20 amino acids of Ha-Ras, which include the CAXX farnesylation motif and the palmitoylation sites (25), was fused to GFP (GFP-CAXX). The localization of GFP-CAXX was very similar to the localization of GFP-Ras. Furthermore, the traffic of GFP-CAXX in response to insulin was identical to that of the full-length protein. In fact, internalized GFP-CAXX was found in endocytic vesicles and colocalized with endogenous Ras (Fig. 2C), indicating that the post-translational modifications of the C terminus are sufficient to target GFP to the correct subcellular localization.

Evanescent wave microscopy was used to further examine the dynamics of GFP-Ras on the plasma membrane. In this technique, an internally reflected laser is used to illuminate a very thin section adjacent to the coverslip, essentially restricting the plane of observation to a thin slice that includes the plasma membrane (26). Two pools of GFP-Ras were detected using this technique. A fraction of plasma membrane-bound GFP-Ras was found localized in punctate microdomains, whereas the bulk of GFP-Ras was found dispersed on the membrane (Fig. 3A). Stimulation with insulin resulted in the disappearance of some of the Ras-containing microdomains (Fig. 3A, arrows). Furthermore, insulin treatment caused a decrease in the total amount of fluorescence associated to the plasma membrane section (Fig. 3B). Control experiments showed that: 1) the total area of the section did not change significantly during the course of experimentation and 2) the excitation protocol did not produce detectable photobleaching. Therefore, we conclude that the decrease in the fluorescence of the plasma membrane was a result of the internalization of Ras and its subsequent movement away from the plane of illumination. This motion is consistent with the formation of endocytic vesicles. To confirm that the disappearance of GFP-Ras from the membrane was a consequence of endocytic traffic, the cells were treated with cytochalasin D, which inhibits endocytosis by destabilizing actin filaments (11, 27). As shown in Fig. 3B, treatment with cytochalasin D inhibited insulin-dependent decrease in plasma membrane associated GFP-Ras, therefore confirming that the decrease in fluorescence intensity of the plasma membrane section is due to the traffic of GFP-Ras during endocytosis.

Because cholesterol is an essential structural component of lipid rafts and of other specific lipid microdomains, drugs that acutely deplete plasma membrane cholesterol and disrupt the structure of these domains have been used to investigate their functional role (28). Therefore, the cholesterol depleting drug, CD, was used to deplete membrane cholesterol in HIRcB cells. The distribution of GFP-Ras on the plasma membrane of CD-treated cells was diffuse, and no punctate structures containing GFP-Ras were observed (Fig. 4A). Furthermore, CD treatment

**Fig. 2.** Agonist-dependent internalization of GFP-Ras. A, the distribution of cholesterol and GFP-Ras in fixed cells was examined by epifluorescence microscopy. Insulin stimulation was for 5 min prior to fixation. B, cells expressing GFP-Ras, GFP-Ras(S17N), or GFP-CAXX were imaged before and after insulin stimulation (200 ns, 5 min). Frames after insulin stimulation are sequential (10 s). Movies are available in the supplemental information. C, the distribution of Ras was examined in insulin-treated (5 min) GFP-CAXX expressing cells by immunostaining and confocal microscopy.
blocked insulin-dependent GFP-Ras internalization. Epifluorescence images showed that CD treatment resulted in a smooth distribution of GFP-Ras on the plasma membrane. Insulin treatment of cholesterol-depleted cells did not promote the traffic of GFP-Ras (Fig. 4B). However, repletion of cellular cholesterol by addition of cholesterol-CD inclusion complexes (19) restored GFP-Ras the internalization, indicating that Ras traffic requires the presence of cholesterol. Taken together, these data support a model in which insulin stimulates endocytosis of lipid microdomains with structural and biological properties akin to those of lipid rafts. Proteins that reside in these domains, such as Ras or the GFP-CAA construct, are subsequently internalized along with the raft.

The role of Ras traffic in the regulation of the Ras-MAPK pathway was then examined. Because Ras binds and activates the serine/threonine kinase Raf-1, we examined the effect of cholesterol depletion on Raf-1 activation. It has been established that Raf-1 must associate with membranes for activation. Cholesterol depletion with CD resulted in accumulation of Raf-1 on the plasma membrane (Fig. 5A) rather than on endosomal membranes (Fig. 5A and Ref. 5). Insulin-dependent Raf-1 activation was not significantly affected by CD treatment (Fig. 5B) as determined by an in vitro kinase assay. Because Raf-1 activation in this system requires interaction with Ras proteins, this indicates that the capacity of Ras to become active and interact with effectors does not require lipid raft-like structures. However, CD treatment inhibited insulin-dependent MEK and MAPK phosphorylation. This result is consistent with a general requirement for endocytosis in agonist-dependent MAPK activation that has been reported in many systems (29–32). Likewise, cytochalasin D also inhibited insulin-dependent MAPK phosphorylation in this system (Fig. 5B), supporting the conclusion that CD treatment inhibits insulin-dependent MAPK phosphorylation through its effects on endocytosis of the Ras-Raf complex.

DISCUSSION

Lipid rafts and other cholesterol-rich microdomains are important structural elements of the membrane that play a role in the regulation of both endocytic and signal transduction processes. According to the classical signal transduction models, following receptor activation, specific signals are passed off to other components that remain attached to the plasma membrane, such as Ras, phosphoinositide 3-kinase and the heterotrimeric G-proteins. Activation of these elements then leads to the stimulation of specific enzymatic activities on the plasma membrane that result in the generation of soluble or membrane-bound second messengers or in the phosphorylation of specific target proteins. Endocytosis, in contrast, was primarily thought to provide signal termination by removing the active receptor from the cell surface and promoting deactivation of the receptor-ligand complex (33). Recently, it has become apparent that endocytosis and signal transduction share many components and may actually be aspects of a single phenomenon. For example, Akt/protein kinase B has been shown to participate in the activation of the endocytic machinery (34), as well as regulating Raf-1 activity (35). Likewise, MAPK has recently been shown to phosphorylate members of the Rab5 subfamily of GTPases (36), which are important regulators of endocytosis.

Here we have provided direct evidence that, during insulin signaling, endocytosis and the activation of the MAPK cascade are coupled. Prior to insulin stimulation, Ha-Ras is distributed between the bulk membrane and cholesterol-rich microdomains that share many of the properties of lipid rafts and...
also Ref. 5). The most relevant feature of these vesicles is that MEK and MAPK appear to be associated to their surface (see X-100. These vesicles contain insulin receptors, and Ras, Raf-1, surface of endocytic vesicles that remain insoluble in Triton Ha-Ras is translocated to an internal pool while attached to the membranes and associate with lipid rafts (20, 39). However, they appear to be essential functional units. This conclusion is derived from the fact that, although functional Ras-Raf-1 complexes may exist on the plasma membrane, phosphorylated MEK is found exclusively on these endosomes. It follows from these observations that the traffic of Ras and Raf-1 is required not for the activation of Raf-1 kinase, but for the interaction of Raf-1 with its main endogenous substrate. The structural and biochemical reasons for this still remain unclear.

The linkage between endocytosis and the MAPK cascade was initially proposed on the basis of the effects of dynamin negative mutants on the activation of the MAPK cascade by receptor tyrosine kinases (41, 42) and G-protein-linked receptors (29, 30). More recent work has suggested that the relationship between receptor endocytosis and the activation of the MAPK cascade is casual rather than causal and that it is the inhibition of the traffic of other cellular components that causes the effects of dynamin (43). The data described here support this hypothesis. Effective receptor-dependent activation of Ras-Raf-1 complexes occurs at the plasma membrane level. However, other components are necessary for the coupling of Raf-1 activation to the phosphorylation of MEK. A plausible explanation for this observation is that the effective coupling of the MAPK cascade is not a new phenomenon (for instance, proteins containing FYVE domains are exclusively recruited to endosomes) (44).

We therefore propose that the effective formation of scaffolding complexes on the surface of endocytic vesicles is a requirement for the coupling of Ras-Raf-1 activation to the phosphorylation of MAPK.

Many of the spatial-temporal features of the recruitment of
cytosolic proteins to the surface of cell membranes are regulated by the appearance and disappearance of lipid second messengers. We have shown, for instance, that Raf-1 binding to membranes is primarily driven by its interactions with phosphatidic acid (5, 6). Coincidentally, a putative scaffolding protein that binds MEK and MAPK, Ksr (the so-called kinase suppressor of Ras) (45, 46), has a sequence that is almost identical to the phosphatidic acid binding domain of Raf-1. Therefore, the model that emerges is one in which phosphatidic acid brings Ksr and Raf-1 into close contact on the surface of endosomes that are enriched in phosphatidic acid, cholesterol, and possibly phosphatidylinositol derivatives. Further work is needed to validate the correctness of this model.

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