Activation of Mitogen-activated Protein Kinase by Membrane-targeted Raf Chimeras Is Independent of Raft Localization*

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Received for publication, May 3, 2001, and in revised form, June 29, 2001
Published, JBC Papers in Press, July 16, 2001, DOI 10.1074/jbc.M103995200

 Binding of proteins to the plasma membrane can be achieved with various membrane targeting motifs, including combinations of fatty acids, isoprenoids, and basic domains. In this study, we investigate whether attachment of different membrane targeting motifs influences the signaling capacity of membrane-bound signal transduction proteins by directing the proteins to different membrane microdomains. We used c-Raf-1 as a model for a signaling protein that is activated when membrane-bound. Three different membrane targeting motifs from K-Ras, Fyn, and Src proteins were fused to the N or C terminus of Raf-1. The ability of the modified Rafs to initiate MAPK signaling was then investigated. All three modified Raf-1 constructs activated MAPK to nearly equivalent levels. The extent of localization of the Raf-1 constructs to membrane microdomains known as rafts did not correlate with the level of MAPK activation. Moreover, treatment of cells with the raft disrupting drug methyl-β-cyclodextrin (MβCD) caused activation of MAPK to levels equivalent to those achieved with membrane-targeted Raf constructs. The use of pharmacological agents as well as dominant negative mutants revealed that MAPK activation by MβCD proceeds via a phosphoinositide 3-kinase-dependent mechanism that is Ras/Raf-independent. We conclude that cholesterol depletion from the plasma membrane by MβCD constitutes an alternative pathway for activating MAPK.

Covalent modification by fatty acylation and prenylation is found on a wide variety of viral and cellular signaling proteins, including protein kinases, guanine nucleotide-binding proteins, transmembrane receptors, and viral structural proteins (1–3).Binding of proteins to the plasma membrane can be achieved with various membrane targeting motifs, including combinations of fatty acids, isoprenoids, and polybasic domains. For example, Src uses myristate with a polybasic stretch to anchor to the plasma membrane; whereas Fyn, a Src family member, uses a myristate + palmitate motif. A C-terminal farneoyl group in conjunction with a polybasic stretch (K-Ras) or palmitates (Ha-Ras) are required to promote membrane binding of Ras proteins (6, 7). It is not clear why such a variety of targeting motifs exist and whether each individual motif promotes a unique function.

Rafts are specialized plasma membrane microdomains that are highly enriched in cholesterol, glycosphingolipids, and glycosphatidylinositol-anchored proteins (8–11). Membrane rafts are relatively resistant to extraction with non-ionic detergents and exhibit a low buoyant density. Recent studies have shown that fatty acylation of proteins, such as Src family kinases and Go subunits, promotes targeting to these membrane microdomains (12–15). Moreover, targeting of proteins to rafts is often required for the initiation of signal transduction. For example, fatty acylation and localization of Lck, Fyn, and LAT (linker for activation of T cells) to rafts are essential for their function in T cell receptor-mediated signal transduction (15–17). Other studies have shown that rafts link multiple receptor tyrosine kinases to mitogen-activated protein kinase (18, 19). Furuchi and Anderson (20) also reported that cholesterol depletion of rafts causes hyperactivation of MAPK.

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* This work was supported by National Institutes of Health Grant GM 57966. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 276, No. 37, Issue of September 14, pp. 34617–34623, 2001

This paper is available online at http://www.jbc.org

Published, JBC Papers in Press, July 16, 2001, DOI 10.1074/jbc.M103995200

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular-regulated protein kinase; MEK, MAPK/ERK kinase; P38, phosphoinositide 3-kinase; MβCD, methyl-β-cyclodextrin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; p-ERK, phospho-ERK.

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Antibodies and Reagents—Rabbit polyclonal anti-ERK2 (c-14), anti-caveolin-1 (N-20), and anti-Raf-1 (c-12) as well as monoclonal anti-c-Myc (9E10), anti-p-ERK (E-4), anti-phosphotyrosine (PY20), and anti-caveolin-1 (N-20), and anti-Raf-1 (c-12) as well as monoclonal anti-c-Myc (9E10), anti-p-ERK (E-4), anti-phosphotyrosine (PY20), and anti-
Ha-Ras were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-pan-Ras was from Calbiochem. Monoclonal anti-Raf-1 was purchased from Transduction Laboratories (Lexington, KY). Rabbit polyclonal anti-MAPK was purchased from New England Biolabs (Beverly, MA). The polyclonal PI3K antibody was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). Fluorescein isothiocyanate-conjugated anti-mouse and rabbit secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Optiprep was purchased from Life Technologies, Inc. Wortmannin, cholerasterol, filipin, and methyl-β-cyclodextrin were purchased from Sigma. LY294002 was purchased from Biomol (Plymouth Meeting, PA). PD98059 was purchased from Calbiochem (San Diego, CA).

**Raf Chimeras and cDNA Constructs—**Myc-Raf was constructed as follows. A sense primer was designed containing a SacI site, start codon, 10-amino acid Myc tag and the N-terminal 6 amino acids of c-Raf. An antisense primer that began 661 bases from start codon of c-raf including a unique HindIII site was constructed. The polymerase chain reaction product generated from these two primers was digested with SacI and HindIII and used to replace the corresponding region of Raf in pGEM-7zf (+). For the Myc-Raf-K-Ras construct, an antisense primer was designed that encoded the 6 C-terminal amino acids of c-Raf-1, followed by the C-terminal 18 amino acids of K-Ras, a stop codon, and an XbaI site and three extra bases. A sense primer was constructed that included a unique EcoRV site upstream from the 5′ end of c-Raf. The polymerase chain reaction product was digested with EcoRV/XbaI and subcloned into Myc-Raf-pGEM-7zf (+) digested with the same enzymes. The construction of Raf-Myc was based on the same methods as those described for Myc-Raf-K-Ras, except the antisense primer contained codons for the 10-amino acid Myc tag. Fusion of the 16 N-terminal Fyn or 20 N-terminal Src amino acids onto the N terminus of c-Raf-1 was accomplished by the methods described for the Myc-Raf construction, except the sense primer encoded Fyn or Src amino acids. All constructs were verified by DNA sequencing prior to subcloning into the pCMV5 vector.

Ha-RasN17 was a gift from Dr. Neal Rosen (Memorial Sloan-Kettering Cancer Center, New York). The pSp5 and p110-CAAX constructs were gifts from Dr. Kurt Ballmer-Hofer (Paul Scherrer Institute, Switzerland).

**Cell Culture and Transfections—**COS-1 cells were maintained as described previously (4). COS-1 cells were transfected with FuGene 6 (Roche Molecular Biochemicals) or LipofectAMINE 2000 (Life Technologies, Inc.) according to the manufacturer’s instructions. Cells were processed at 48–72 h after transfection.

**Cell Lysis and Western Blotting—**Cells were lysed in RIPA buffer (150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% deoxycholic acid, 1% Triton X-100, 10 mM Tris pH 7.4, 1 mM Na3VO4, 10 μg/ml leupeptin, 10 μg/ml aprotinin) and harvested in STE with a rubber policeman. Cell pellets were obtained by centrifugation at 1,000 × g for 5 min at 4 °C. The cell pellet was resuspended in 300 μl of TNET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.5% Triton X-100) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin), incubated on ice for 20 min, and then adjusted to 35% Optiprep. Cell extracts were overlaid successively with 3.5 ml of 30% Optiprep in TNET and 200 μl of TNET, respectively, in SW55 tubes. Samples were centrifuged for 4 h at 170,000 × g at 4 °C, and five fractions were collected from the top of the gradient. Each fraction was adjusted to 1× RIPA buffer and clarified at 100,000 × g for 4 °C for 15 min. Proteins from each fraction were analyzed by SDS-PAGE (either SDS-PAGE or by direct SDS-PAGE and Western blotting).

**RESULTS**

**Generation of Membrane-bound Chimeric Raf Constructs—**To test whether individual membrane-binding motifs have unique functions in signal transduction, we constructed forms of Raf that were modified by the addition of three different membrane-binding motifs (Fig. 1A). The prenylated tail from K-Ras was appended to the C terminus of Raf, whereas the myristate + basic and myristate + palmitate sequences from Src and Fyn, respectively, were appended to the N terminus of Raf. A Myc epitope tag was added to each construct in order to distinguish the recombinant Raf molecules from endogenous Raf-1. Subcellular fractionation was performed to examine whether Raf constructs tagged with the different membrane-binding motifs were targeted to membranes, by separating transfected COS-1 cells into cytosolic (S100) and membrane (P100) fractions. As depicted in Fig. 1, A and B, Raf molecules containing the various membrane-binding motifs were localized predominantly to the membrane fraction. In contrast, Raf-Myc and Myc-Raf were mainly present in the cytosolic fraction. This demonstrates that the appended membrane-binding signals were functional in the context of Raf chimeras.

**Chimeric Raf Constructs Are Targeted to the Plasma Membrane—**In order to examine whether the Raf constructs tagged with various membrane-binding motifs can target to the plasma membrane, COS-1 cells transfected with the Raf constructs were subjected to immunofluorescence staining with either anti-Raf or anti-Myc antibodies. The Raf-Myc and Myc-Raf proteins were mainly cytoplasmic, whereas the three Raf constructs tagged with the different membrane-targeting motifs exhibited a typical plasma membrane staining pattern (Fig. 2).

**Membrane-targeted Raf Constructs Induce Strong Levels of ERK Activation—**Previously, it was shown that Raf modified with the K-Ras membrane-binding motif can activate ERK independent of Ras. Here, we compared the biological effects of the three tagged Raf constructs on ERK. Activation of ERK was quantitated by Western blotting with anti-p-ERK antibody (which recognizes only activated ERK) and normalized to the total amount of ERK and Raf construct in each set of transfected cells. The three Raf constructs modified with different membrane-binding domains all caused a more than 10-fold increase in ERK activity, as shown in Fig. 3. Both Myc-Raf and Raf-Myc caused only a slight increase in ERK activity above the background level. These data demonstrate...
motifs from the following proteins: Fyn, myristate/H11001, Myc epitope at either the N or C terminus, and the membrane-targeting A, Ras-Raf-MAPK signal transduction pathway, was also analyzed. Ha-RasG12V and K-RasG12V, which play an important role in caveolae, was used as a positive control. As expected, the majority of Raf constructs served as negative controls; these unmodified proteins were found to be excluded completely from rafts. Caveolin-1, an endogenous protein marker for lipid rafts and proteins present in the P100 membrane fraction as well as the S.D. are shown on the right side of A.

that plasma membrane targeting of Raf is sufficient to activate ERK, consistent with the current model that activation of Raf is due to the recruitment to the plasma membrane by activated Ras (21, 22).

Raft Localization of the Raf-1 Constructs—We next examined the localization of the various modified Raf constructs to rafts. Optiprep density gradient centrifugation was used to separate raft-associated proteins from detergent-sensitive proteins. COS-1 cells transfected with the various Raf constructs were harvested and extracted in ice-cold buffer containing 0.5% Triton X-100. After flotation on Optiprep gradients, five fractions were collected from top to bottom. Rafts are located in the top fraction at the 0/30% interface. The distribution of Raf proteins was analyzed for activation of ERK by SDS-PAGE and Western blotting with anti-p-ERK antibody. As depicted in Fig. 4C, both Ras proteins were almost completely excluded from rafts, consistent with previous studies (26). Moreover, neither ERK nor p-ERK partitioned into raft fractions. Taken together, these results indicate that the extent of raft localization of the Raf constructs did not directly correlate with the level of ERK activation shown in Fig. 3.

Cyclodextrin Activates ERK—We next used the raft disrupting drug MβCD to examine further a potential role of rafts in activation of ERK. COS-1 cells transfected with the various Raf constructs were treated with MβCD for 1 h at 37 °C. Cell lysates were analyzed for activation of ERK by SDS-PAGE and Western blotting with anti-p-ERK antibody. As depicted in Fig. 5A, MβCD had a striking effect on ERK activity, causing a nearly 10-fold increase in p-ERK in mock-transfected, Myc-Raf and Raf-Myc-transfected cells. A similar result was obtained in NIH 3T3 cells, where addition of MβCD caused a 10-fold activation of ERK (Fig. 5B). In cells expressing the membrane-targeted Raf proteins, there was little to no additional activation of ERK in the presence of MβCD (Table I). Levels of total ERK were the same in all samples.

In order to determine whether the effects of MβCD were related to the ability of this compound to bind cholesterol, COS-1 cells were incubated with MβCD in the presence or absence 40 μg/ml cholesterol. As depicted in Fig. 5C, cholesterol addition prevented ERK activation by MβCD, with the amount of p-ERK reduced to near basal levels. Moreover, addition of another cholesterol-binding agent, filipin, also caused a 4–5-fold activation of ERK (Fig. 5D). These data imply that cholesterol depletion from cell membrane induces ERK activation.

Activation of ERK by MβCD Is Ras-independent—The next set of experiments addressed the mechanism by which ERK is activated by MβCD. A dominant negative Ras (Ha-RasN17) construct was used to examine whether activation of ERK by MβCD is dependent on Ras. As depicted in Fig. 6, Ha-RasN17 had no effect on activation of ERK induced by MβCD. In con-
trast, Ha-RasN17 almost completely inhibited activation of ERK mediated by constitutively activated Ha-Ras (Ha-RasG12V). These results indicate that M/H9252CD-mediated ERK activation occurs through a Ras-independent pathway.

Activation of ERK by M/H9252CD Is PI3K-dependent—Previous studies have indicated a critical role for PI3K in activation of Ras and ERK by epidermal growth factor (27, 28). Here, we investigated whether M/H9252CD-induced activation of ERK requires PI3K activity. COS-1 cells were incubated with or without the PI3K inhibitor wortmannin during M/H9252CD treatment. Phosphorylation of endogenous ERK was visualized using anti-p-ERK immunoblotting. Pretreatment with wortmannin almost completely abolished the activation of ERK induced by MβCD, as shown in Fig. 7A. However, wortmannin had no effect on activation of ERK induced by Myc-Raf-K-Ras. The role of PI3K in MβCD-stimulated ERK activation was further confirmed by utilizing LY294002, another inhibitor of PI3K, which is structurally and mechanistically different from wortmannin. As shown in Fig. 7B, treatment of cells with LY294002 inhibited MβCD-induced activation of ERK in a dose-dependent manner. To confirm the data obtained with pharmacological inhibitors, we examined the ability of Δp85, a dominant negative PI3K mutant lacking the p110-binding site, to inhibit MβCD-mediated ERK activation. The activation of ERK was ~50% (±10%, n = 4) lower when Δp85 was expressed, compared with mock-transfected cells (Fig. 7C). This level of inhibition correlates with a transfection efficiency of 43% (±6%, n = 6) obtained in these experiments. Finally, activation of PI3K by MβCD was assessed directly, by quantitating the extent of tyrosine phosphorylation of endogenous p85. Phos-
MAPK Activation by Membrane-targeted Raf

Effect of MβCD on ERK activity mediated by modified Raf constructs

COS-1 cells transfected with the indicated Raf constructs were treated with 1% MβCD for 1 h at 37 °C. Following cell lysis, activated ERK was detected by Western blotting with a p-ERK-specific antibody. Total levels of ERK did not change significantly during the treatment. Activation of ERK in the absence of MβCD was normalized to 1 in pCMV5-transfected cells. The graph shows activation of ERK after MβCD treatment based on quantitations of data from two independent experiments. B. NIH 3T3 cells were incubated in the absence or presence of 1% MβCD for 24 h, and ERK activation was monitored as described in A.

C, COS-1 cells were untreated (1st lane), treated with 1% MβCD for 1 h at 37 °C (2nd lane), or treated with 1% MβCD and 40 μg/ml cholesterol for 1 h at 37 °C (3rd lane). ERK activation was monitored as described in A. D. COS-1 cells were incubated in the absence or presence of 10 μg/ml filipin, and ERK activation was monitored as described in A.

| Constructs | ERK activity |
|------------|--------------|
|            | −MβCD | +MβCD | S.D.   |
| Myc-Raf-K-Ras | 1    | 1.38  | ±0.10  |
| Fyn-Raf-Myc  | 1    | 1.22  | ±0.04  |
| Src-Raf-Myc  | 1    | 1.20  | ±0.25  |

Fig. 5. MβCD activates ERK. A, COS-1 cells transfected with the indicated Raf-1 constructs were treated with 1% MβCD for 1 h at 37 °C. Following cell lysis, activated ERK was detected by Western blotting with a p-ERK-specific antibody. Total levels of ERK did not change significantly during the treatment. Activation of ERK in the absence of MβCD was normalized to 1 in pCMV5-transfected cells. The graph shows activation of ERK after MβCD treatment based on quantitations of data from two independent experiments. B. NIH 3T3 cells were incubated in the absence or presence of 1% MβCD for 24 h, and ERK activation was monitored as described in A. C, COS-1 cells were untreated (1st lane), treated with 1% MβCD for 1 h at 37 °C (2nd lane), or treated with 1% MβCD and 40 μg/ml cholesterol for 1 h at 37 °C (3rd lane). ERK activation was monitored as described in A. D. COS-1 cells were incubated in the absence or presence of 10 μg/ml filipin, and ERK activation was monitored as described in A.

Table I

Activation of ERK by MβCD Is Partially Dependent on MEK—We next investigated the role of MEK in MβCD-mediated ERK activation. COS-1 cells were incubated with or without 20 μM PD98059 (a specific MEK inhibitor) for 1 h at 37 °C during MβCD treatment. Subsequently, the cells were lysed, and activation of ERK was analyzed by using anti-p-ERK antibody immunoblotting. As shown in Fig. 8, the activation of ERK by MβCD was inhibited by about 50% in the presence of PD98059. In contrast, activation of ERK by Ha-RasG12V and Myc-Raf-K-Ras was nearly completely inhibited by PD98059. All samples contained approximately equal amounts of total ERK. Thus, although MEK is essential for both Ha-RasG12V- and Myc-Raf-K-Ras-mediated ERK activation, activation of ERK by MβCD is only partially dependent on MEK activity.

DISCUSSION

In this paper, we have examined the role of membrane microdomains in activation of MAPK by plasma membrane-bound Raf. There is growing evidence in the literature to indicate that concentration of signaling proteins in membrane microdomains such as rafts and/or caveolae is essential for efficient signal transduction. For example, localization of Src family kinases and LAT to cholesterol, sphingolipid-enriched rafts is required for activation of downstream signaling via the activated T cell receptor (15–17). Other reports have suggested that localization of receptor tyrosine kinases to caveolae is important for stimulation of the MAPK pathway (18, 19). c-Raf-1, a key intermediate in this pathway, has been reported to be recruited to caveolae upon activation of the epidermal growth factor receptor or Ha-RasG12V (18).

Therefore, we tested the role of membrane microdomains by quantitating the ability of three different membrane-targeted Raf-1 constructs to activate MAPK. The amount of p-ERK was normalized to the total amount of each Raf-1 construct and to the total amount of ERK in each set of transfected cells. We found that the extent of MAPK activation by the Src-, Fyn-, and K-Ras-modified Raf proteins was essentially equivalent. It
should be noted that expression of an Ha-Ras-tagged Raf construct resulted in 2–3-fold less p-ERK than the other tagged constructs (data not shown). However, indirect immunofluorescence analysis revealed that, in addition to the plasma membrane, a significant amount of Myc-Raf-Ha-Ras was localized to intracellular perinuclear membranes. Perinuclear staining of full-length Ha-Ras, as well as other Ha-Ras-tagged chimeras, has been observed previously and reflects the trafficking of these proteins through the endoplasmic reticulum and Golgi (25). Thus, the lower level of p-ERK in these cells may be due to the fact that there is less Myc-Raf-Ha-Ras at the plasma membrane, compared with the other tagged constructs.

The extent of MAPK activation did not correlate with the extent of raft localization of the Raf chimeras. As depicted in Fig. 4, the Src and Fyn motifs efficiently targeted the chimeric Raf proteins to rafts, whereas Raf tagged with the prenylated tail from K-Ras was excluded from rafts. The behavior of the chimeric proteins described here mimics that of the full-length proteins; Src and Fyn are enriched in rafts (14, 19), whereas Ras proteins are largely excluded from rafts (see below) (26). The raft fractions that were isolated are heterogeneous and include detergent-resistant membranes as well as caveolae. We conclude that, once Raf is membrane-bound, the activation of ERK is raft-independent.

The physiological upstream activator of Raf is Ras, which serves to recruit Raf to the plasma membrane. There is now increasing evidence in the literature to indicate that Ras isoforms differ in their abilities to activate downstream effectors. For example, K-Ras activates Raf-1 to a much greater extent than Ha-Ras, whereas Ha-Ras activation of PI3K is greater than that achieved by K-Ras (29). Moreover, Ha-Ras and K-Ras are localized to different regions of the plasma membrane. K-Ras is distributed throughout the bulk plasma membrane, and activation of Raf by K-Ras is independent of membrane microdomain structure or function. In contrast, the inactive form of Ha-Ras is localized to rafts. Upon binding of GTP, Ha-Ras is released from rafts, a step that is required for full activation of Raf (30, 31). Taken together, these data indicate that Ras-dependent activation of Raf occurs less efficiently in rafts, but once Raf is constitutively membrane-bound, its activation is raft-independent.

MβCD is a water-soluble cyclic oligomer that sequesters cholesterol within its hydrophobic core (32). It has been extensively used as an agent to deplete cholesterol from the plasma membrane of cultured cells (33). Here we show that MβCD treatment of COS-1 cells results in a dramatic stimulation of MAPK, as reflected in a nearly 10-fold increase in the amount of p-ERK. Stimulation of MAPK by MβCD had been noted previously (20), but the mechanism of activation had not been addressed. In this work, we utilized a combination of pharmacological inhibitors and dominant negative mutants to examine the mechanism of MAPK activation by MβCD. We show that the MβCD effect on MAPK is Ras-independent, as expression of dominant negative Ras had no effect on p-ERK formation in the presence of MβCD (Fig. 6). The MβCD effect occurred downstream or independently from Raf. No additive effect was observed when cells expressing membrane-targeted Raf constructs were treated with MβCD (Table I), and MβCD has no direct effect on Raf activity (30).
Several lines of evidence support the hypothesis that MβCD activates MAPK via a PI3K-dependent pathway. Treatment of cells with the PI3K inhibitors wortmannin and LY294002 inhibited MβCD-induced activation of MAPK (Fig. 7). Moreover, MAPK activation by MβCD was inhibited when cells were transfected with a dominant negative PI3K mutant, and a 3-fold increase in tyrosine-phosphorylated p85 was observed in MβCD-treated cells. Previous studies have established a close correlation between p85 phosphorylation and PI3K activity (43). Activation of PI3K, however, was not sufficient to activate MAPK, as no effect of p110-C, a constitutively activated mutant of PI3K, was observed on p-ERK formation (data not shown). Moreover, MEK was partially required, as concentrations of the MEK-specific inhibitor PD98059 that completely blocked Ras-G12V activation of MAPK had only partial (50%) inhibitory effects on MβCD activation of MAPK (Fig. 8).

At least four mechanisms for activation of MAPK by PI3K-dependent pathways have been reported. Generation of inositol phospholipids by activated PI3K has been shown to stimulate protein kinase C, which then stimulates Raf and activates MAPK (34, 35). PI3K has been shown to activate Rac, which activates MEK via stimulation of p21-activated kinase and Raf (36). Recently, Yart et al. (28) reported that MAPK activation in epidermal growth factor-stimulated Vero cells was PI3K-dependent; the mechanism involved recruitment of SHP2 and the adaptor protein Gab, upstream of Ras. Finally, G protein-coupled receptors, through Gβγ, have been shown to recruit PI3K to the membrane. This results in activation of Src and the use of an SHC-Grb2-SOS complex to activate Ras and ultimately MAPK (37, 38). Since MβCD activation of MAPK is independent of Ras (Fig. 6), Raf (30), and Src (data not shown), it is unlikely that any of the above pathways are involved in the PI3K-dependent activation of MAPK by MβCD.

Rather, we favor the hypothesis that the MβCD activation of MAPK is a consequence of the depletion of plasma membrane cholesterol. The cholesterol-depleting agent filipin also activated MAPK (Fig. 5D). The MβCD effect on MAPK was blunted by adding back cholesterol (Fig. 5C), as has also been shown by Furuchi and Anderson (20). Cholesterol levels in cells are sensed by sterol regulatory element-binding proteins (39), and it will be interesting to determine whether these endoplasmic reticulum-localized proteins are involved in the MβCD effect on MAPK. Alternatively, PI3K (40) and MAPK (41) have been shown to be present on lipid droplets, cytoplasmic storage sites for triacylglycerides, and cholesterol (42). It is tempting to speculate that cholesterol depletion by MβCD might regulate signal transduction by these cytoplasmic organelles. Taken together, the data presented in the current study indicate that depletion of cholesterol from the cell plasma membrane results in a proliferative response and that MβCD has effects on cells beyond disruption of raft and caveolae structure.

Acknowledgments—We thank Xiquan Liang and Wolf Lindwasser for helpful discussions, Raya Louft-Nisenbaum for technical assistance, and Debra Alston for secretarial support.

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J. Biol. Chem. 2001, 276:34617-34623.
doi: 10.1074/jbc.M103995200 originally published online July 16, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M103995200

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