

Cholesterol Depletion from the Plasma Membrane Triggers Ligand-independent Activation of the Epidermal Growth Factor Receptor*

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Xu Chen and Marilyn D. Resh‡

From the Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and Graduate Program in Biochemistry and Structural Biology, Weill Graduate School of Medical Sciences of Cornell University, New York, New York 10021

We recently demonstrated that depletion of plasma membrane cholesterol with methyl- β -cyclodextrin (M β CD) caused activation of MAPK (Chen, X., and Resh, M. D. (2001) *J. Biol. Chem.* 276, 34617–34623). MAPK activation was phosphatidylinositol 3-kinase (PI3K)-dependent and involved increased tyrosine phosphorylation of the p85 subunit of PI3K. We next determined whether M β CD treatment induced tyrosine phosphorylation of other cellular proteins. Here we report that cholesterol depletion of serum-starved COS-1 cells with M β CD or filipin caused an increase in Tyr(P) levels of a 180-kDa protein that was identified as the epidermal growth factor receptor (EGFR). Cross-linking experiments showed that M β CD induced dimerization of EGFR, indicative of receptor activation. Reagents that block release of membrane-bound EGFR ligands did not affect M β CD-induced tyrosine phosphorylation of EGFR, indicating that M β CD activation of EGFR is ligand-independent. Moreover, M β CD treatment resulted in increased tyrosine phosphorylation of EGFR downstream targets and Ras activation. Incubation of cells with the specific EGFR inhibitor AG4178 blocked M β CD-induced phosphorylation of EGFR, SHC, phospholipase C- γ , and Gab-1 as well as MAPK activation. We conclude that cholesterol depletion from the plasma membrane by M β CD causes ligand-independent activation of EGFR, resulting in MAPK activation by PI3K and Ras-dependent mechanisms. Moreover, these studies reveal a novel mode of action of M β CD, in addition to its ability to disrupt membrane rafts.

Cholesterol is a membrane lipid that regulates both the flexibility and the mechanical stability of the membrane bilayer. It has also been shown that cholesterol plays a critical role in assembling membrane microdomains, such as rafts and caveolae, in a separate phase from the rest of the bilayer (1–3). Cholesterol and lipid rafts are involved in numerous cellular processes, including membrane protein segregation and concentration, protein and lipid sorting, and virus assembly and release (1, 3, 4). However, excess cholesterol is toxic to cells and can contribute to the development of diseases such as atherosclerosis and Alzheimer's disease (5, 6). It is therefore impor-

tant for cells to maintain cholesterol homeostasis.

Methyl- β -cyclodextrin (M β CD)¹ is a water-soluble cyclic heptasaccharide that has been used to deliver hydrophobic drugs based on its property of solubilizing non-polar substances (7). This compound has also been demonstrated to bind cholesterol with high specificity (7). Cholesterol from the plasma membrane of cultured cells is rapidly removed in response to M β CD (8–10), and M β CD has therefore been extensively used as a cholesterol-depleting reagent. Recent studies also indicate that cholesterol depletion by M β CD disrupts membrane rafts (11, 12) and affects signaling pathways at the cell surface (13, 14).

We recently demonstrated (15) that cholesterol depletion by M β CD induced ERK activation via a PI3K-dependent pathway. How cholesterol removal regulated this pathway was not clear. We had observed that treatment of cells with M β CD caused an increase in tyrosine phosphorylation of the p85 subunit of PI3K. It was therefore of interest to determine whether cholesterol depletion by M β CD induces tyrosine phosphorylation of other cellular proteins. Here we show that M β CD treatment of cells induced tyrosine phosphorylation of multiple proteins. A combination of Tyr(P) antibody/agarose affinity purification and mass spectrometry was used to identify one of the proteins (180 kDa) as the epidermal growth factor receptor (EGFR). M β CD treatment induced tyrosine phosphorylation of downstream targets of EGFR, including SHC, PLC- γ , and Gab-1 as well as Ras activation. We conclude that cholesterol depletion from the plasma membrane triggers signal transduction through ligand-independent activation of the EGF receptor.

MATERIALS AND METHODS

Antibodies and Reagents—Goat polyclonal anti-p-EGFR (Tyr-1173), rabbit polyclonal anti-EGFR (1005), anti-ERK2 (C-14), and anti-Gab-1 (H-198), and monoclonal anti-Tyr(P) (PY99) and anti-p-ERK (E-4) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-EGFR (neutralizing) and monoclonal anti-PLC- γ were obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-p-EGFR (Tyr-1068) and anti-p-EGFR (Tyr-845) were purchased from Cell Signaling Technology (Beverly, MA). Rabbit polyclonal anti-SHC and mouse monoclonal anti-SHC were obtained from BD Biosciences. Fluorescein isothiocyanate-conjugated anti-mouse secondary antibody was obtained from Molecular Probes (Eugene, OR). GM6001 was obtained from Chemicon International (Temecula, CA). Bis(sulfosuccinimidyl) suberate (BS³) was obtained from Pierce, Warrmanning, TPA, CRM197, cholesterol, filipin, and methyl- β -cyclodextrin were purchased from Sigma. Tyrphostin AG1478 was obtained from Biomol (Plymouth Meeting, PA). Epidermal growth factor was obtained from Calbiochem. Trappsol was obtained from Cyclodextrin Technologies Development Inc. (High Springs, FL).

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‡ To whom correspondence should be addressed: Cell Biology Program, Memorial Sloan-Kettering Cancer Center, 1275 York Ave., Box 143, New York, NY 10021. Tel.: 212-639-2514; Fax: 212-717-3317; E-mail: m-resch@ski.mskcc.org.

¹ The abbreviations used are: M β CD, methyl- β -cyclodextrin; EGF, epidermal growth factor; EGFR, EGF receptor; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3-kinase; PBS, phosphate-buffered saline; BS³, bis(sulfosuccinimidyl) suberate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PLC, phospholipase C.

Cell Culture, Transfection, and Cholesterol Depletion—COS-1 cells, A431 cells, and 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (COS-1, A431) or with 10% calf serum (3T3 cells). COS-1 cells were transfected with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions. Cholesterol depletion of cells was performed as described previously (15). Briefly, cells were serum-starved for 24 h and then incubated with the indicated concentration of M β CD (dissolved in Dulbecco's modified Eagle's medium) or other indicated cholesterol depletion reagents for 1 h at 37 °C before lysis. For inhibitor experiments, serum-starved cells were incubated for 1 h at 37 °C with indicated inhibitors in the presence of 2% M β CD prior to harvest.

Identification of Proteins by Mass Spectrometry—Six 100-mm plates of COS-1 cells were serum-starved and then treated with 2% M β CD for 1 h. Cells were lysed in RIPA buffer (150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% deoxycholate, 1% Triton X-100, 10 mM Tris, pH 7.4, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 250 μ g/ml Pefabloc (Roche Molecular Biochemicals)) and then subjected to affinity purification using an anti-Tyr(P)/agarose conjugate. Samples were washed three times with RIPA buffer and analyzed by SDS-PAGE and Coomassie Blue staining. Individual protein bands were excised and digested with trypsin, and the tryptic peptides were subjected to mass fingerprinting by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry by the Sloan-Kettering Microchemistry Facility. Top "major" experimental masses (m/z) were used to search a non-redundant human data base using the PeptideSearch algorithm. A molecular weight range twice the predicted weight was covered, with a mass accuracy restriction of 40 ppm or better, and a maximum of one missed cleavage site allowed per peptide. In addition, mass spectrometric based sequencing (electrospray ionization-mass spectrometry) of selected peptides was performed using a PE-SCIEX API300 triple quadrupole instrument, fitted with a continuous flow nano-electrospray source ("JaFIS").

Immunoprecipitation and Immunoblotting—Cells were lysed with RIPA buffer, and lysates were clarified at 14,000 rpm in an Eppendorf centrifuge for 10 min at 4 °C. For immunoprecipitation, lysates were incubated with the indicated antibodies and protein A-agarose (Santa Cruz Biotechnology). Samples were washed three times with RIPA buffer and analyzed by SDS-PAGE. Western blotting was performed using the indicated antibodies. Proteins were detected using horseradish peroxidase-conjugated secondary antibodies and ECL Western blotting detection reagents following the manufacturer's instructions. Ras activation was assayed using a Ras Activation Assay Kit (Upstate Biotechnology) following the manufacturer's instructions.

Immunofluorescence Microscopy—Immunofluorescence staining was carried out as described previously (16). COS-1 cells were seeded onto 25-mm glass coverslips and serum-starved for 24 h before treatment with 1% M β CD for 2 h. The cells were washed twice with PBS, fixed with 3.7% formalin/PBS for 15 min, permeabilized with 0.2% Triton in PBS for 5 min, and incubated with anti-Tyr(P) monoclonal antibody for 45 min. Cells were washed four times with PBS and incubated with fluorescein isothiocyanate-conjugated secondary antibody for 30 min. Following four 5-min washes, cells were mounted on microscope slides and visualized with a Zeiss LSM510 confocal microscope.

Cross-linking Studies—Cross-linking experiments were performed as described (17) with minor modification. COS-1 cells were grown to subconfluency and then serum-starved for 24 h. Cells were treated with 2% M β CD for 1 h at 37 °C or as indicated, then transferred to room temperature, and washed twice in phosphate-buffered saline (PBS) buffer. The membrane-impermeable cross-linker bis(sulfosuccinimidyl) suberate (BS³) was dissolved in PBS and added to a final concentration of 2 mM for 15 min at room temperature. The reaction was quenched for 5 min by the addition of 1 M glycine, pH 8.0 (final concentration of 50 mM). Cells were washed with cold PBS and lysed in buffer (50 mM HEPES, pH 7.5, 10% glycerol, 0.5% Triton X-100, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM Na₃VO₄, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 250 μ g/ml Pefabloc), and the lysate was clarified at 14,000 rpm in an Eppendorf centrifuge for 10 min at 4 °C. Supernatants were adjusted to SDS-PAGE sample buffer containing 5% β -mercaptoethanol and subjected to SDS-PAGE on a 5% polyacrylamide gel, followed by immunoblotting with anti-EGFR polyclonal antibody.

Measurement of Cholesterol Content in the Plasma Membrane Fraction—COS-1 cells grown in 100-mm culture dishes were treated with or without 2% M β CD for 1 h. The plasma membrane fraction was isolated as described previously (18) and suspended in a minimum of PBS. An aliquot was used for protein measurement. The rest of the pellet was extracted with 2:1 methanol/chloroform, followed by 0.5 ml of chloroform and 0.5 ml of water. The chloroform (lipid phase) was dried under

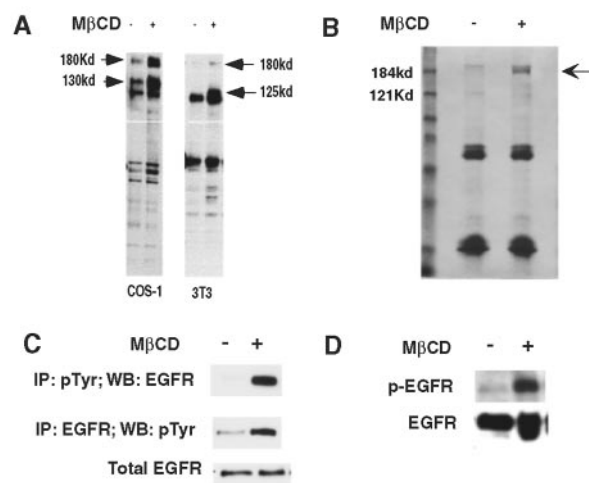


FIG. 1. M β CD stimulates tyrosine phosphorylation of the EGFR. A, serum-starved COS-1 and NIH 3T3 cells were preincubated with or without 1% M β CD for 1 h and lysed. The lysates were subjected to SDS-PAGE followed by Western blotting with anti-Tyr(P) antibody. B, serum-starved COS-1 cells were incubated in the absence or presence of 2% M β CD for 1 h at 37 °C. Cells were lysed and subjected to affinity purification using an anti-Tyr(P)/agarose conjugate. The samples were analyzed by SDS-PAGE and Coomassie Blue staining. The 180-kDa protein band was excised and analyzed by mass spectrometry as described under "Materials and Methods," resulting in identification of the p180 protein as EGF receptor. C, serum-starved COS-1 cells were stimulated with or without 2% M β CD for 1 h. The cell lysates were immunoprecipitated (IP) with the indicated antibodies followed by Western blotting (WB) with the indicated antibodies. D, serum-starved A431 cells were incubated in the absence or presence of 2% M β CD for 1 h. The cell lysates were analyzed by Western blotting with an anti-pEGFR antibody.

nitrogen. The dry lipid was suspended in 2-propanol, and membrane cholesterol was assayed using the Sigma Infinity cholesterol reagent according to the manufacturer's instructions.

RESULTS

M β CD Induces Tyrosine Phosphorylation of Proteins in COS-1 Cells—Our recent studies (15) showed that treatment of COS-1 cells with M β CD activated MAPK via a PI3K-dependent pathway. Moreover, M β CD-treated cells exhibited enhanced tyrosine phosphorylation of the p85 subunit of PI3K. We therefore examined whether tyrosine phosphorylation of other cellular proteins was increased in response to M β CD. COS-1 cells were serum-starved for 24 h and then were treated with 1% M β CD for 1 h at 37 °C. Cell lysates were analyzed by SDS-PAGE and anti-Tyr(P) Western blotting. As shown in Fig. 1A, tyrosine phosphorylation levels of two prominent proteins, termed p180 and p130, were increased upon treatment with M β CD. Similar results were also observed in NIH 3T3 cells. Furthermore, immunofluorescence staining of COS-1 cells with anti-Tyr(P) antibody revealed a striking increase in the levels of cellular protein tyrosine phosphorylation upon treatment of M β CD, compared with control cells, particularly at the plasma membrane and in the perinuclear region (data not shown). These results demonstrate that M β CD can induce tyrosine phosphorylation of cellular proteins.

Identification of Tyrosine-phosphorylated Proteins Stimulated by M β CD—The next set of experiments was designed to identify the M β CD-induced phosphorylated proteins. Serum-starved COS-1 cells were treated with 2% M β CD for 1 h, and cell lysates were subjected to affinity purification using an anti-Tyr(P)/agarose conjugate. Bound protein samples were resolved by SDS-PAGE and Coomassie Blue staining. As shown in Fig. 1B, an 180-kDa band was apparent in the lane from M β CD-treated cells. The p180 band was excised and digested with trypsin. Mass spectrometric fingerprinting analysis was

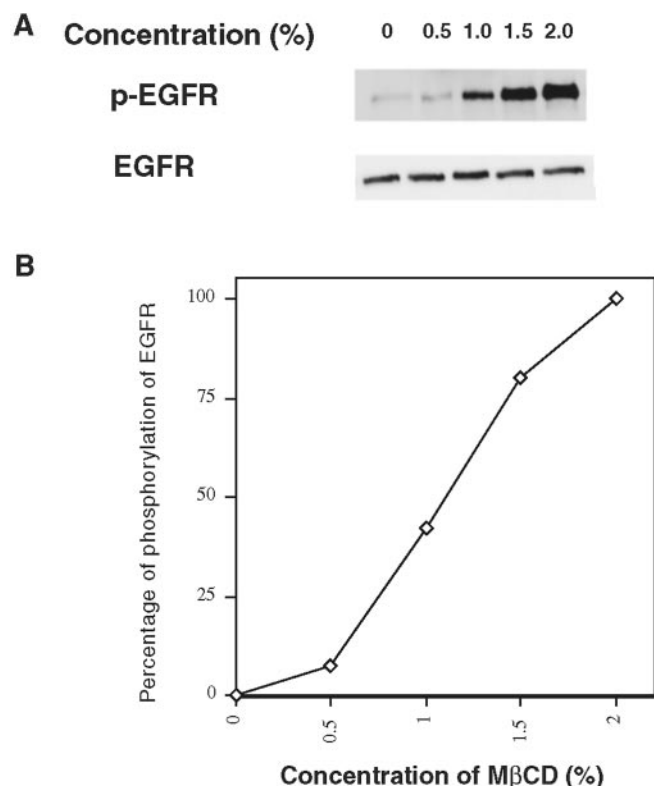


FIG. 2. Concentration-dependent effects of MβCD on tyrosine phosphorylation of EGFR. A, serum-starved COS-1 cells were incubated in the presence of the indicated concentrations of MβCD for 1 h. Cell lysates were subjected to SDS-PAGE and Western blotting with an anti-pEGFR antibody. B, quantitation of data from the above experiment is plotted. Phosphorylation of EGFR stimulated by 2% MβCD was normalized to 100%.

performed as described under "Materials and Methods," resulting in the identification of p180 as the EGFR.

To confirm that Tyr(P) 180 was the EGFR, immunoprecipitation and Western blotting of lysates from MβCD-treated COS-1 cells was performed with anti-EGF receptor and anti-Tyr(P) antibodies. As depicted in Fig. 1C, treatment of cells with MβCD clearly induced EGFR phosphorylation. Similar results were also observed using an anti-p-EGFR antibody, which recognizes only tyrosine-phosphorylated (Tyr-1173) EGFR. In human A431 cells, which express high levels of EGFR protein, MβCD strongly increased EGFR phosphorylation (Fig. 1D). Moreover, the MβCD-induced increase in EGFR phosphorylation was inhibited by AG4178, a specific inhibitor of EGFR tyrosine kinase activity (19). It is important to note that these experiments were performed with serum-starved cells, implying that phosphorylation of EGFR induced by MβCD is ligand-independent (see below).

Role of Cholesterol in MβCD-induced EGFR Phosphorylation—We next examined the concentration dependence of MβCD on EGFR phosphorylation. Serum-starved COS-1 cells were treated with increasing concentrations of MβCD for 1 h at 37 °C and then lysed. Samples were subjected to SDS-PAGE and Western blotting using an anti-pEGFR antibody. MβCD induced phosphorylation of EGFR in a concentration-dependent manner (Fig. 2), with maximal EGFR phosphorylation occurring at 2% MβCD. Concentrations of MβCD above 2% resulted in loss of cell viability.

Quantitation of cholesterol levels revealed that addition of 2% MβCD resulted in an ~40% reduction in membrane cholesterol content (Fig. 3A). In order to determine whether depletion of cholesterol by MβCD was responsible for EGFR phosphorylation,

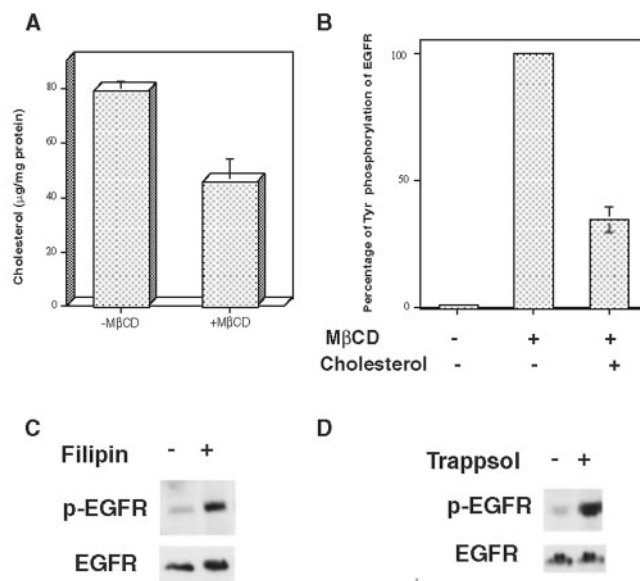


FIG. 3. The role of cholesterol in tyrosine phosphorylation of EGFR induced by MβCD. A, COS-1 cells were treated with or without 2% MβCD for 1 h and washed, and the plasma membrane fraction was isolated. The amount of cholesterol in the membrane fraction was measured by using Sigma Infinity Cholesterol Reagent (see "Materials and Methods"). Data are expressed as the amount of cholesterol per mg of total plasma membrane protein. B, 1% MβCD was preincubated with or without 40 μg/ml cholesterol and added to serum-starved COS-1 cells for 1 h. Cell lysates were subjected to SDS-PAGE followed by Western blotting using an anti-pEGFR antibody. Phosphorylation of EGFR was analyzed and quantitated from three independent experiments. The level of phosphorylation of EGFR induced by MβCD was normalized to 100%. C and D, serum-starved COS-1 cells were incubated in the absence or presence of 10 μg/ml filipin (C) or 40 mM Trappsol (D). Phosphorylation of EGFR was monitored as described in B.

ation, MβCD was preincubated with or without 40 μg/ml cholesterol and then added to cells for 1 h. The effect of cholesterol on MβCD-induced EGFR phosphorylation was then assessed. As shown in Fig. 3B, cholesterol addback inhibited MβCD-induced EGFR phosphorylation by 60–70%.

Next we examined whether other cholesterol depletion agents can cause ligand-independent EGFR phosphorylation. COS-1 cells were incubated with 2-OH-propyl-β-cyclodextrin (Trappsol), which is as effective in depleting cell membrane cholesterol as MβCD (20), or filipin, a cholesterol-binding agent (21). Both Trappsol and filipin caused EGFR phosphorylation (Fig. 3, C and D). These data imply that cholesterol depletion from the cell membrane induces EGFR phosphorylation.

MβCD Causes Ligand Independent Dimerization and Activation of EGFR—It has been established previously that upon EGF binding, EGFR undergoes ligand-induced dimerization, a prerequisite for normal receptor signaling (22). The tyrosine phosphorylation of EGFR induced by MβCD occurs in the absence of EGF addition. We therefore investigated whether MβCD can also cause EGFR dimerization, indicative of EGFR activation. Serum-starved COS-1 cells were stimulated with 2% MβCD for 1 h at 37 °C and then exposed to the membrane-impermeable cross-linker BS³. As shown Fig. 4, MβCD increased dimerization of EGFR to a similar extent as 25 ng/ml EGF, implying that MβCD induced ligand-independent activation of EGFR.

Previous studies (23–25) have reported that transactivation of EGFR can occur by release of membrane-anchored EGFR ligands; this event is mediated by activation of transmembrane metalloproteinases. We therefore performed two sets of experiments to determine whether MβCD activation of EGFR was truly ligand-independent. First, cells were treated with a neu-

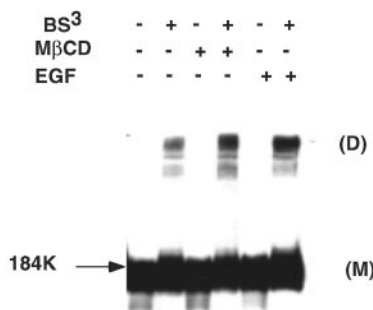


FIG. 4. Ligand-independent dimerization of EGFR. Serum-starved COS-1 cells were incubated in the absence or presence of 2% MβCD for 1 h or 25 ng/ml EGF for 3 min at 37 °C. Cell-surface receptors were cross-linked in the presence of 2 mM BS³, a membrane-impermeable cross-linker, for 15 min at room temperature. Cell lysates were subjected to SDS-PAGE gels (5% polyacrylamide) followed by Western blotting with anti-EGFR antibody. The positions of EGFR dimer (D) and monomer (M) are indicated.

tralizing antibody that effectively blocks the ligand-binding site of the EGFR. As depicted in Fig. 5A, antibody addition nearly completely blocked phosphorylation of the EGFR by EGF but had no effect on EGFR phosphorylation induced by MβCD. Second, addition of the broad spectrum metalloproteinase inhibitors GM6001 (Fig. 5B) or BB-94 (not shown) had no effect on MβCD-induced EGFR activation but blocked EGFR transactivation induced by TPA. A similar result was obtained with CRM197, a diphtheria toxin mutant that specifically blocks the action of HB-EGF (26). TPA-induced transactivation of EGFR was inhibited by CRM197, whereas no effect was observed in MβCD-treated cells (Fig. 5B). Thus, MβCD-induced activation of EGFR does not involve release of membrane-bound ligands.

An alternative possibility was that MβCD activates a Src-dependent EGFR phosphorylation and transactivation process (27). Treatment of cells with MβCD followed by Western blotting with phospho-specific EGFR antibodies revealed increases in tyrosine phosphorylation of two major autophosphorylation sites Tyr-1173 and Tyr-1068 as well as Tyr-845, a site phosphorylated by Src (Fig. 5C). Addition of PP2, a Src kinase inhibitor, caused a partial (about 50%) inhibition of MβCD-induced tyrosine phosphorylation of Tyr-845. However, PP2 had no effect on the MβCD-induced increase in pERK (Fig. 5D), indicating that Src was not likely to be involved in MAPK activation by MβCD.

Inhibition of EGFR Blocked MβCD-induced ERK Activation—It is well established that binding of EGF to EGFR causes ERK activation (22). We therefore investigated whether ligand-independent phosphorylation of EGFR induced by MβCD is responsible for ERK activation. Serum-starved COS-1 cells were incubated with or without the specific EGFR inhibitor AG4178 during MβCD treatment. The levels of p-EGFR and p-ERK were then analyzed. Pretreatment with AG4178 almost completely blocked both EGFR phosphorylation and ERK activation induced by MβCD as well as EGF (Fig. 6). These data demonstrate that EGFR phosphorylation induced by MβCD mediates ERK activation.

MβCD-induced Tyrosine Phosphorylation of EGFR Targets and Ras Activation—We next investigated whether ligand-independent EGFR activation by MβCD caused tyrosine phosphorylation of the EGFR targets SHC, PLC-γ, and Gab-1. Lysates from serum-starved COS-1 cells that were not treated or were treated with MβCD were immunoprecipitated with anti-SHC or PLC-γ or Gab-1 antibodies, followed by SDS-PAGE and Western blotting with an anti-Tyr(P) antibody. As shown in Fig. 7, treatment of cells with MβCD induced tyrosine phos-

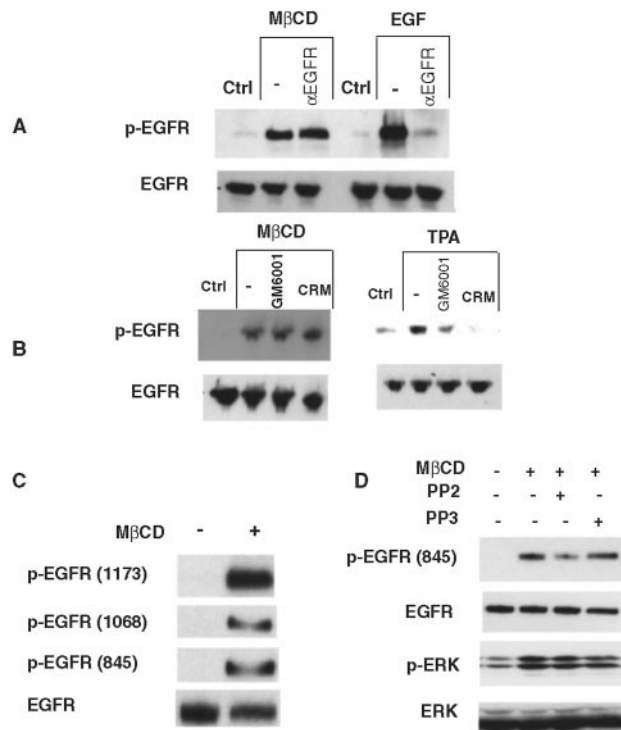


FIG. 5. EGFR phosphorylation induced by MβCD is ligand-independent. A and B, serum-starved COS-1 cells were pretreated with neutralizing antibodies (10 μg/ml) against EGFR (αEGFR) or 5 mM metalloproteinase inhibitor GM6001 or 10 μg/ml HB-EGF inhibitor CRM197 for 30 min and then treated with 2% MβCD for 1 h (in the presence of above-mentioned antibody or inhibitor) or 50 ng/ml EGF for 3 min or 600 ng/ml TPA for 3 min. Cell lysates were subjected to SDS-PAGE and Western blotting with the indicated antibodies. Ctrl., control. C, serum-starved COS-1 cells were incubated in the absence or presence of 2% MβCD for 1 h. Cell lysates were subjected to SDS-PAGE and immunoblotting with phospho-specific anti-EGFR antibodies to the indicated phosphotyrosine residue or with anti-EGFR antibody. D, serum-starved COS-1 cells were incubated in the absence or presence of 2% MβCD and/or 5 μM PP2 or 5 μM PP3 (inactive analog of PP2) for 1 h. Cell lysates were analyzed by Western blotting using the indicated antibodies.

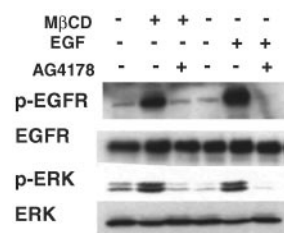


FIG. 6. EGFR is required for ERK activation induced by MβCD. Serum-starved COS-1 cells were incubated in the absence or presence of 0.5 μM EGFR kinase inhibitor AG4178 for 1 h in the presence or absence of MβCD. For positive control experiments, serum-starved COS-1 cells were preincubated with 0.5 μM AG4178 for 1 h and then stimulated with 25 ng/ml EGF for 3 min. After cell lysis, the protein samples were subjected to SDS-PAGE followed by Western blotting using the indicated antibodies.

phorylation of all three SHC isoforms (p66, p52, and p46) as well as PLC-γ and Gab-1. MβCD-induced SHC, PLC-γ, and Gab-1 phosphorylation levels were reduced by about 90% in the presence of the EGFR inhibitor AG4178 (data not shown). Thus, ligand-independent activation of EGFR results in phosphorylation of downstream targets of EGFR.

In order to monitor Ras activation, cells were treated with MβCD or EGF, and the amount of activated Ras was quantitated using a Ras binding domain-glutathione S-transferase pull-down assay. As depicted in Fig. 8, 2% MβCD induced a

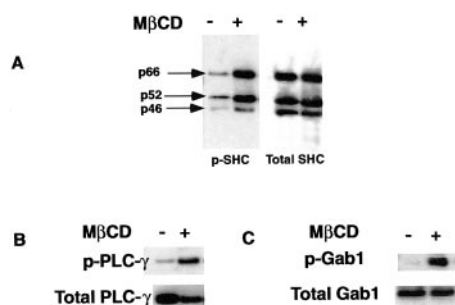


FIG. 7. M β CD stimulates SHC, PLC- γ and Gab-1 tyrosine phosphorylation. Serum-starved COS-1 cells were incubated in the absence or presence of 2% M β CD for 1 h. Cell lysates were immunoprecipitated with anti-SHC (A), or anti-PLC- γ (B), or anti-Gab-1 (C) antibody followed by Western blotting with an anti-Tyr(P) antibody. The total amounts of SHC, PLC- γ , and Gab-1 were not affected by M β CD. The experiment shown is representative of 2–3 similar independent experiments.

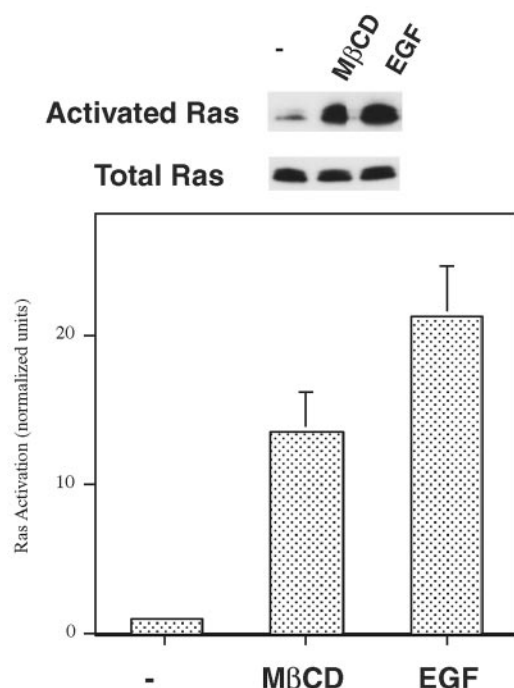


FIG. 8. M β CD activates Ras. Serum-starved COS-1 cells were incubated in the absence or presence of 2% M β CD for 1 h or 25 ng/ml EGF for 3 min. Cell lysates were precipitated with Raf-1 Ras binding domain-conjugated agarose, followed by SDS-PAGE and Western blotting with anti-Ras antibody. The amount of activated Ras was quantitated. Data shown are representation of four independent experiments.

10–13-fold activation of Ras, compared with 20-fold obtained with 25 ng/ml EGF. It is therefore likely that Ras-dependent pathways contribute to ERK activation by M β CD.

The Role of PI3K in M β CD-induced EGFR Signaling—Our previous study (15) established that M β CD induced ERK activation in a PI3K-dependent manner. We therefore performed experiments to determine at which stage in the signaling pathway PI3K was involved. The PI3K inhibitor wortmannin had no effect on M β CD-induced tyrosine phosphorylation of EGFR, SHC, PLC- γ , and Gab-1 (data not shown). However, wortmannin did significantly inhibit M β CD-induced ERK activation. Wortmannin caused a 50% reduction in pERK levels in cells treated with 2% M β CD and a 75% reduction in pERK in cells treated with 1% M β CD (Fig. 9, A and B). In order to determine whether a Ras/Raf/MEK pathway was responsible for the residual pERK activity, we incubated M β CD-treated cells in the presence, absence, or combination of wortmannin and/or

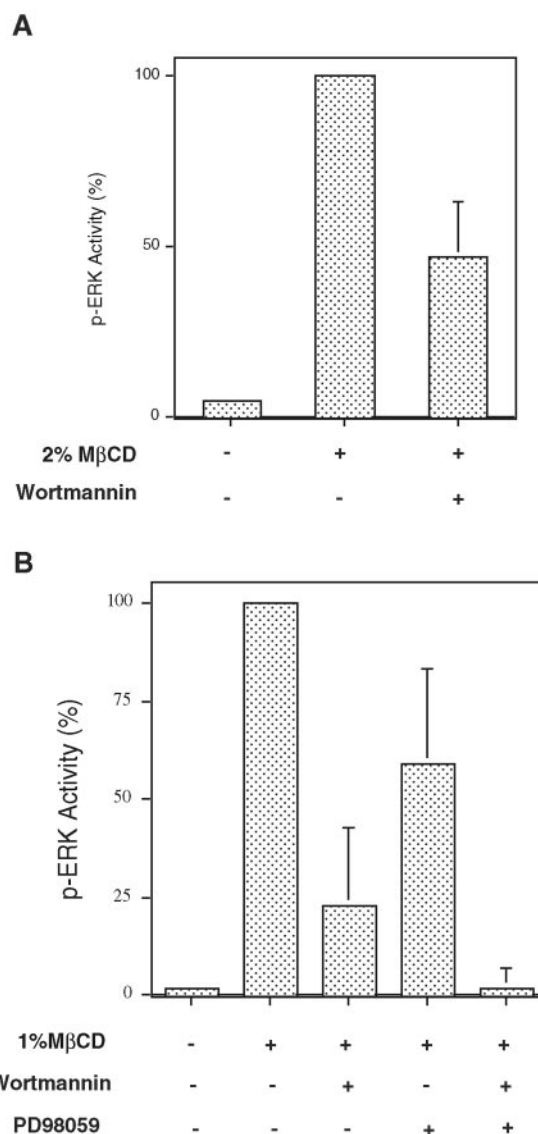


FIG. 9. M β CD activates MAPK by PI3K and Ras-dependent pathways. Serum-starved COS-1 cells were incubated in the absence or presence of 2% M β CD (A) or 1% M β CD (B) with or without 200 nM wortmannin or 20 μ M PD98059. Following cell lysis, activated ERK was detected by Western blotting with a pERK-specific antibody and was normalized to the total amount of ERK. Data was quantitated from 2 to 3 independent experiments.

PD98059, a MEK inhibitor. As depicted in Fig. 9B, the combination of wortmannin and PD98059 reduced pERK to basal levels. Taken together, these results suggest that both PI3K and Ras-dependent pathways contribute to the M β CD-induced activation of EGFR signaling.

DISCUSSION

Cyclodextrins such as M β CD effectively remove cholesterol from the plasma membrane (8–10, 14, 28, 29). This property has made M β CD an extensively used agent to study the function of rafts, membrane microdomains whose integrity depends on the presence of cholesterol. In this report, we show that cholesterol depletion by M β CD also has a striking effect on tyrosine phosphorylation of endogenous cellular proteins. The data presented here clearly demonstrate that M β CD treatment results in activation of the endogenous EGFR, as evidenced by increased tyrosine phosphorylation and dimerization of EGFR, increased tyrosine phosphorylation of downstream substrates of EGFR, including endogenous SHC, PLC γ , and Gab-1, and

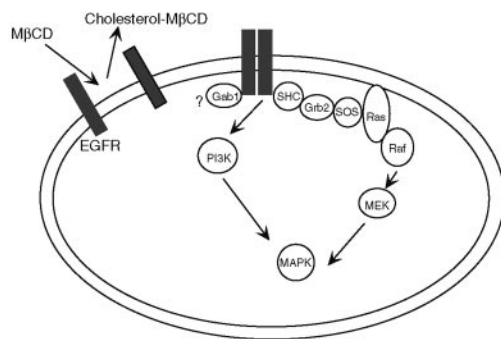


FIG. 10. **A model for M β CD induced MAPK activation.** Depletion of plasma membrane cholesterol by M β CD leads to dimerization and activation of EGFR and signaling via PI3K and Ras-dependent pathways. See text for details.

activation of ERK. All of these events were blocked by AG4178, a specific EGFR kinase inhibitor, implying that M β CD induced EGFR signal transduction.

Several lines of evidence support the hypothesis that depletion of plasma membrane cholesterol plays a critical role in M β CD-mediated EGFR activation. First, multiple cholesterol-binding reagents, including M β CD, filipin, and trappsol, caused EGFR phosphorylation (Fig. 3, C and D). Moreover, the M β CD effect on EGFR phosphorylation was blunted by preincubating M β CD with cholesterol (Fig. 3B). Based on these results we conclude that cholesterol depletion by M β CD triggers EGFR signaling.

The data presented in the current study, combined with our recent report (15), establish a mechanism whereby M β CD induces ERK activation, *i.e.* activation of EGFR. Moreover, this work sheds light on the signaling pathways that are induced as a result of M β CD-induced EGFR activation. Multiple lines of evidence implicate PI3K as being required for M β CD-induced ERK activation. For example, expression of a dominant-negative PI3K mutant blocks M β CD-induced ERK activation (15). In addition, the adaptor protein Gab-1, which links activated growth factor receptors to PI3K (30, 31), is tyrosine-phosphorylated in M β CD-treated cells (Fig. 7C). Moreover, both wortmannin and LY294002, specific PI3K inhibitors, reduce ERK activation by M β CD (15). It is interesting to note that the extent of inhibition by wortmannin varied with the concentration of M β CD, with 75% inhibition of ERK activation obtained when cells were treated with 1% M β CD, and only 50% inhibition at 2% M β CD (Fig. 9). These results imply that additional, PI3K-independent pathways are activated by increasing M β CD concentrations.

We observed previously (15) that transfection of cells with dominant-negative Ha-RasN17 had no apparent effect on M β CD-induced ERK activation. However, limitations posed by transfection efficiency and/or contributions from other Ras isoforms may have obscured a potential role for Ras in this system. We therefore utilized a more sensitive and specific assay, which detects binding of activated Ras to the Ras binding domain of Raf (32). The data depicted in Fig. 8 reveal that Ras is indeed activated by M β CD, and it is likely that Ras activation also contributes to pERK formation.

A model that illustrates our current knowledge of M β CD-induced ERK activation is depicted in Fig. 10. Treatment of cells with M β CD causes cholesterol depletion from the plasma membrane, EGFR dimerization and activation, and EGFR autophosphorylation. At least two downstream signaling pathways are then activated. Phosphorylation and recruitment of SHC triggers the classical Ras/Raf/MEK/MAPK cascade. In addition, PI3K is activated, potentially through Gab-1 (30, 31), leading to MAPK activation via a still unknown mechanism

(33). Inhibiting either pathway, with wortmannin or PD98059, provides only partial inhibition of M β CD-induced ERK activation, whereas in the presence of both inhibitors, pERK is reduced to basal levels (Fig. 9). Thus, the Ras and PI3K pathways apparently operate independently and in parallel.

Alterations in Protein Phosphorylation Induced by M β CD—M β CD-triggered increases in protein phosphorylation have also been observed in other studies. For example, M β CD treatment induced transient tyrosine phosphorylation of ZAP-70, LAT, and phospholipase γ 1 in T cells (13) and resulted in constitutive phosphorylation of SHC in rat adipocytes (14). Removal of plasma membrane cholesterol by M β CD was shown to increase tyrosine phosphorylation in sperm (34). Moreover, cholesterol depletion by M β CD has been shown to hyperactivate ERK and increase EGFR phosphorylation (28, 35, 36). The mechanisms responsible for induction of protein phosphorylation by M β CD in these systems have not been identified. It will be interesting to determine whether activation of growth factor receptors represents a common signaling mechanism in response to membrane cholesterol levels.

The effects of M β CD on ERK are likely to be dependent on cell type. ERK activation in response to M β CD has been observed in COS-1 (15), NIH 3T3 (15), PC12 (37), Rat-1 (28), and T lymphocytes (13, 38). In contrast, M β CD inhibits the activation of ERK induced by shear stress or ischemia in endothelial cells (39, 40) or by insulin in HIRcB cells (41). However, in rat adipocytes, cholesterol depletion by β -cyclodextrin has no effect on ERK activation (14). The reason for these differences in cellular responses is not known but may be related to differences in EGFR abundance.

Ligand-independent Activation of the EGFR—Activation of EGFR signaling in the absence of exogenously added ligand has been observed in several systems. For example, deletions in the extracellular ligand-binding domains of the EGFR have been found in several human tumors, including gliomas, as well as in the avian retroviral oncoprotein vErbB. These mutants trigger oncogenic signals in a ligand-independent manner (42, 43). Transactivation of EGFR can be induced by G-protein-coupled receptors (44, 45) or platelet-derived growth factor (46). In addition, oxidative stress or expression of E-cadherins in epithelial cells stimulates activation of EGFR and subsequent activation of MAPK (47, 48). In many of the instances cited above, EGFR activation has been shown to be mediated by stimulated release of membrane-anchored EGF-like ligand precursors (23–25, 49, 50).

EGFR signaling induced by M β CD occurred in the absence of exogenous EGF, suggesting that ligand-independent activation of EGFR was occurring. Several lines of evidence support the conclusion that EGFR activation by M β CD is truly ligand-independent. First, it is unlikely that M β CD induced the synthesis of EGF, resulting in autocrine stimulation. When conditioned media from cells treated with M β CD were placed on serum-starved cells, no activation of ERK and EGFR phosphorylation was observed (data not shown). Second, treatment with a neutralizing monoclonal antibody, which effectively blocks the ligand-binding site of the EGFR, blocked the action of exogenously added EGF but had no effect on M β CD-induced EGFR phosphorylation and activation (Fig. 6). Third, addition of broad spectrum matrix metalloproteinase inhibitors GM6001 or BB-94 had no effect on M β CD-induced EGFR phosphorylation and activation but blocked EGFR transactivation by TPA (Fig. 6). Finally, the Src inhibitor PP2 had no effect on M β CD-induced ERK activation, implying that Src-dependent transactivation of EGFR was not contributing to signaling. Taken together, these data strongly support the hypothesis that cholesterol depletion triggers ligand-independent EGFR activation.

Several potential mechanisms may account for the ability of M β CD to induce EGFR activation. It has been shown recently (51, 52) that cholesterol depletion by M β CD inhibits clathrin-coated budding and prevents formation of clathrin-coated endocytic vesicles. Therefore, one possibility is that endocytosis of EGFR is inhibited by M β CD, resulting in a higher concentration of EGFR at the cell surface. Burke *et al.* (53) have provided evidence that EGFR signaling is regulated by endocytosis and intracellular trafficking. However, it has been shown that signal transduction from internalized EGFR also occurs from endosomes (54). For example, Oksvold *et al.* (55) showed that a substantial fraction of tyrosine-phosphorylated EGFR and Shc, Grb2, and pERK exists in endosomes and that EGFR signaling is not only limited to the plasma membrane but also occurs in the early endosome and late endosome.

Alternatively, perturbation of gross membrane structure may mediate EGFR activation. For example, Zwick *et al.* (56) demonstrated that plasma membrane depolarization triggers EGFR activation. Moreover, cholesterol depletion by M β CD has been shown to induce the formation of large scale domains in living cell membranes (29). It is tempting to speculate that ligand-independent EGFR activation by cholesterol depletion may occur in response to formation of these new plasma membrane domains. Finally, two recent reports showed that cholesterol may directly affect EGFR kinase activity. Incubation with water-soluble cholesterol caused decreased EGF-induced EGFR tyrosine phosphorylation, suggesting that the presence of cholesterol negatively regulates EGFR kinase activity (35). Moreover, cholesterol depletion was shown to increase the intrinsic tyrosine kinase activity of the EGFR in membranes generated from M β CD-treated NIH 3T3 cells (36).

The results obtained with M β CD *in vitro* are likely to have potential physiological significance for cholesterol depletion *in vivo*. Millions of patients are currently being treated with statins to lower serum cholesterol. There is increasing evidence in the literature that statin treatment also reduces membrane cholesterol levels (57, 58). It is therefore possible that the effects on signal transduction observed with acute M β CD treatment of cells may be mimicked by long term statin treatment. Although future work is required to elucidate the exact mechanism of cholesterol in regulating EGFR signaling, the data presented in the current study indicate that cholesterol depletion triggers ligand-independent EGFR activation and intracellular signaling.

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