# Cholesterol Levels Modulate EGF Receptor-Mediated Signaling by Altering Receptor Function and Trafficking†

Linda J. Pike* and Laurieann Casey‡

*Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, 660 South Euclid Avenue, Box 8231, St. Louis, Missouri 63110*

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ABSTRACT: A variety of signal transduction pathways including PI turnover, MAP kinase activation, and PI 3-kinase activation have been shown to be affected by changes in cellular cholesterol content. However, no information is available regarding the locus (or loci) in the pathways that are susceptible to modulation by cholesterol. We report here that depletion of cholesterol with methyl-$\beta$-cyclodextrin increases cell surface $^{125}$I-EGF binding by ~40% via a mechanism that does not involve externalization of receptors from an internal pool. Cholesterol depletion also enhances in vivo EGF receptor autophosphorylation 2−5-fold without altering the rate of receptor dephosphorylation. In vitro kinase assays, which are done under conditions where phosphotyrosine phosphatases are inhibited and receptor trafficking cannot occur, demonstrate that treatment with methyl-$\beta$-cyclodextrin leads to an increase in intrinsic EGF receptor tyrosine kinase activity. EGF receptors are localized in cholesterol-enriched lipid rafts but are released from this compartment upon treatment with methyl-$\beta$-cyclodextrin. These data are consistent with the interpretation that localization to lipid rafts partially suppresses the binding and kinase functions of the EGF receptor and that depletion of cholesterol releases the receptor from lipid rafts, relieving the functional inhibition of the receptor. Cholesterol depletion also inhibits EGF internalization and down-regulation of the EGF receptor, and this likely contributes to the enhanced ability of EGF to stimulate downstream signaling pathways such as the activation of MAP kinase.

The EGF[1] receptor is a member of the family of receptor protein tyrosine kinases. It is an intrinsic membrane protein of 1186 amino acids (*1*). The extracellular ligand binding domain comprises the first 620 amino acids. Residues 647−1186 comprise the intracellular domain and contain the tyrosine kinase module. Between these two domains lies the transmembrane domain that passes through the membrane once in the form of an α helix.

Numerous studies have demonstrated that the EGF receptor is localized to cholesterol-enriched membrane domains such as caveolae or lipid rafts (*2*−*5*). Several studies have suggested that receptor tyrosine kinase-mediated signaling emanates from these low-density membrane domains. Pike and Casey (*6*) demonstrated that EGF-stimulated PI turnover utilized only the pool of phosphatidylinositol 4,5-bisphosphate that was localized to the caveolar fraction of cells. Mineo et al. (*3*) reported that Ras was localized to caveolar fractions and that addition of EGF to cells induced the recruitment of Raf-1 into caveolae. For the related PDGF receptor, both PDGF-stimulated tyrosine phosphorylation (*7*) and MAP kinase activation (*8*) appear to be localized largely to low-density membrane fractions. Thus, growth factor receptor signaling seems to involve cholesterol-enriched membrane domains.

Cholesterol is important for the function of receptor-mediated signaling. Depletion of cholesterol from A431 cells by treatment with methyl-$\beta$-cyclodextrin led to the inhibition of EGF-stimulated PI turnover (*4*). Similarly, treatment of 3T3 cells with lovastatin to reduce cellular cholesterol levels inhibited PDGF-stimulated PI 3-kinase activity (*9*). By contrast, cholesterol depletion of Rat-1 cells led to an enhancement of EGF-stimulated MAP kinase activity (*10*). Thus, depending on the downstream signaling pathway examined, cholesterol depletion appears to have both positive and negative effects on receptor tyrosine kinase-mediated signaling.

The studies cited above document the effects of cholesterol depletion on complex signaling events involving numerous molecules. They do not provide information regarding which components of the signaling pathways were affected by changes in cholesterol content. The current work focuses on the effects of cholesterol depletion on the EGF receptor itself. We report here that cholesterol depletion by treatment with methyl-$\beta$-cyclodextrin results in the enhancement of EGF binding and receptor autophosphorylation and an increase in intrinsic receptor kinase activity as assessed in in vitro assays. This was associated with the loss of EGF receptors from the low-density, lipid raft fraction of cells. These data are consistent with the interpretation that EGF receptor function is suppressed when the receptor is localized to lipid rafts. Cholesterol depletion also decreased the rate of receptor internalization and down-regulation. These findings provide a molecular explanation for the previously observed enhancement of EGF-stimulated MAP kinase activity in cholesterol-depleted cells.

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* To whom correspondence should be addressed. Phone: (314) 362-9502. FAX: (314) 362-7183. E-mail: pike@biochem.wustl.edu.

‡ Current address: Department of Biomolecular Chemistry, University of Wisconsin, Madison, WI 53706.

[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGF, epidermal growth factor; PBS, phosphate-buffered saline.

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## EXPERIMENTAL PROCEDURES

### *Materials*

Methyl-$\beta$-cyclodextrin was purchased from Aldrich. Cholesterol was from Sigma. EGF was purified from mouse submaxillary glands (*11*). The anti-phosphotyrosine antibody PY-20 and the monoclonal anti-flotillin antibody were from Transduction Laboratories (Lexington, KY). The anti-phospho-MAP kinase monoclonal antibody and anti-MAPK polyclonal antibody were from Upstate Biotechnology (Lake Placid, NY). Anti-EGF receptor polyclonal antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) or were generated by immunization of rabbits with a portion of the intracellular domain of the EGF receptor (*12*). Enhanced Chemiluminescence blotting detection reagents were from Amersham Life Sciences. Sulfo-NHS-SS-biotin and streptavidin−agarose were from Pierce. The CII Cholesterol Assay Kit was from Wako. [$\gamma$-$^{32}$P]ATP was from New England Nuclear. $^{125}$I-EGF was synthesized using the chloramine T method as described by McFarthing (*13*). The synthetic tyrosine-containing peptide Arg-Arg-Src (RRLIEDAEY-AARG) was synthesized and purified at the Protein and Nucleic Acid Chemistry Laboratory, Washington University.

### *Methods*

*Cells and Cell Culture.* NIH 3T3 cells expressing wild-type EGF receptors (*12*) were maintained at 37 °C and 5% $CO_2$ in DMEM containing 10% fetal calf serum. Most experiments were performed using cells grown in 60-mm dishes and treated with 3 mL of medium containing methyl-$\beta$-cyclodextrin or cholesterol/methyl-$\beta$-cyclodextrin complexes.

*Treatment with Methyl-$\beta$-cyclodextrin or Sterol/Methyl-$\beta$-cyclodextrin Complexes*. Cells were switched to DMEM containing 0.1% fetal calf serum 16 h before use. For treatment with methyl-$\beta$-cyclodextrin, cells were transferred to DMEM containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin, and 7.5 mM methyl-$\beta$-cyclodextrin. Cultures were incubated at 37 °C for 30 min with occasional swirling. For treatment with cholesterol/methyl-$\beta$-cyclodextrin complexes, cells were incubated for 30 min at 37 °C in DMEM containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin, and the indicated concentration of cholesterol/methyl-$\beta$-cyclodextrin complex. Synthesis of the cholesterol/methyl-$\beta$-cyclodextrin complexes was carried out exactly as described by Klein et al (*14*).

*Cholesterol Assays*. To assess the uptake of cholesterol, cellular lipids were extracted according to the method of Bligh−Dyer (*15*). After evaporating to dryness, the extracts were assayed for cholesterol using the Wako CII Cholesterol Assay Kit that measures total cholesterol levels.

*Stimulation of Cells and Preparation of Lysates*. Cultures were treated with 25 nM EGF for the indicated time and washed once in ice-cold PBS. Cells were lysed in RIPA buffer (150 mM NaCl, 10 mM Tris, pH 7.2, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) containing 20 mM *p*-nitrophenyl phosphate, 100 $\mu$M sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1 $\mu$g/mL leupeptin. After incubation on ice for 10 min with periodic vortexing, lysates were centrifuged for 10 min at 17 000 rpm in a Sorvall RC5B centrifuge using an SS34 rotor. Aliquots of the lysates were assayed for protein. Equivalent amounts of protein from each lysate were mixed with SDS sample buffer and analyzed by SDS−polyacrylamide gel electrophoresis.

*Immunoprecipitations*. RIPA lysates (300 $\mu$g) were incubated for 2 h at 4 °C with 5 $\mu$L of DB-1, a polyclonal anti-EGF receptor antibody (*12*). Complexes were collected by the addition of Pansorbin followed by centrifugation. After three washes in RIPA buffer, SDS sample buffer was added to the pellet which was then boiled for 3 min. Pansorbin was sedimented, and the supernatant was applied to an SDS gel.

*Western Blotting and Detection of Proteins*. Proteins from SDS gels were electrophoretically transferred to nitrocellulose, and the nitrocellulose blots were blocked with 10% powdered milk. Blots were incubated with primary antibody for 2 h, washed, and incubated with the appropriate secondary antibody for 45 min. Proteins were detected using Enhanced Chemiluminescence.

*$^{125}$I-EGF Binding*. Cells were plated in 6-well dishes and incubated in DMEM containing 0.1% serum for 16 h prior to use. For Scatchard analysis, cells were transferred to DMEM containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin, 25 pM $^{125}$I-EGF, and increasing concentrations of unlabeled EGF. Cultures were incubated for 2 h at 4 °C and washed 3 times with ice-cold PBS. Cells were dissolved in 1 mL of 1 N NaOH and counted for $^{125}$I. Data were analyzed using the LIGAND computer program (*16*). For $^{125}$I-EGF internalization, cells were incubated in DMEM containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin, 2 nM $^{125}$I-EGF in the absence or presence of 50 nM unlabeled EGF for the times indicated. One set of cells was washed 3 times in ice-cold PBS and used to determine total binding. A replicate set of cells was washed twice for 2 min in 50 mM glycine, 250 mM NaCl, pH 4.0, and once with PBS and used to determine internalized $^{125}$I-EGF. Specific binding was defined as the difference between binding in the absence and presence of 50 nM unlabeled EGF. Surface binding was calculated as the difference between total binding and internal ligand.

*Membrane Preparation and in Vitro Kinase Assays*. After treatment with or without methyl-$\beta$-cyclodextrin, cells were lysed by homogenization in 25 mM HEPES, pH 7.2, 1 mM PMSF, 1 mM EDTA, 2 $\mu$g/mL leupeptin, 1 $\mu$g/mL pepstatin, and 1 $\mu$g/mL $\alpha$2-macroglobulin. Membranes were pelleted by centrifugation for 15 min at 17 000 rpm in a Sorvall RC5 centrifuge and resuspended in 70 mM $\beta$-glycerophosphate, 200 mM NaCl, 10% glycerol plus protease inhibitors as above. Assays were done in a final volume of 50 $\mu$L containing the following: 50 mM $\beta$-glycerophosphate, 100 $\mu$M ATP, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM *p*-nitrophenyl phosphate, 100 $\mu$M sodium orthovanadate, $1 \times 10^6$ cpm of [$\gamma$-$^{32}$P]ATP, and 15 $\mu$g of membrane protein (*17*). When included, EGF was added at a final concentration of 25 nM, and Arg-Arg-Src peptide was added at a final concentration of 2 mM. Membranes were incubated with growth factor for 20 min on ice prior to the addition of the other assay components. Assays were started by the addition of ATP and metal ions and were incubated for 5 min at 30 °C. For autophosphorylation reactions, incubations were stopped by the addition of 50 $\mu$L of sample buffer. The samples were boiled, run on a 10% polyacrylamide gel, and

Western-blotted using anti-phosphotyrosine antibodies. For peptide phosphorylation assays, incubations were stopped by the addition of 50 $\mu$L of 10% trichloroacetic acid. Precipitated material was pelleted by centrifugation, and 75 $\mu$L of the supernatant was applied to small squares of phosphocellulose paper. The papers were washed in 75 mM $H_3PO_4$ for 30 min with three changes of buffer. After drying, the papers were counted for $^{32}P$ in a liquid scintillation counter.

*Cell Surface Biotinylation*. Cells grown in 60-mm dishes were treated with or without 7.5 mM methyl-$\beta$-cyclodextrin for 30 min at 37 °C. Subsequently, cultures were washed 3 times in warmed PBS and incubated in 2.5 mL of NHS-SS-biotin in PBS (1.5 mg/mL) for 60 min at 4 °C. Cells were rinsed once in PBS containing 50 mM glycine and then incubated in the same buffer for 20 min at 4 °C. After being washed twice in PBS, cells were lysed in 300 $\mu$L of the same buffer containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 $\mu$g/mL leupeptin, 1 $\mu$g/mL pepstatin, and 1 mM benzamidine. After 30 min at 4 °C, lysates were clarified by centrifugation. One hundred microliters of the lysate was applied to 15 $\mu$L of streptavidin−agarose beads and incubated for 1.5 h at 4 °C. The flow-through was applied to another 15 $\mu$L of streptavidin−agarose beads and incubated for 1.5 h. This second flow-through was retained for analysis. The agarose beads were combined, washed 4 times in PBS, and boiled twice for 10 min in 50 $\mu$L of 2-fold concentrated SDS sample buffer. The beads were washed twice in 50 $\mu$L of running buffer, and the washes were combined with the two eluates. The entire eluate, along with 100 $\mu$L of the starting lysate and 100 $\mu$L of the second flow-through, was analyzed by SDS−polyacrylamide gel electrophoresis and Western blotting for EGF receptor.

*Preparation of Lipid Rafts*. Lipid rafts were prepared according to the method of Smart et al. (*18*). Briefly, plasma membranes were isolated by cenrifugation in a Percoll gradient. Purified plasma membranes were sonicated and low-density membranes separated by centrifugation in a 10−20% gradient of Opti-Prep. Gradients were fractionated into 1.2 mL fractions, and equal volumes of each fraction were analyzed by Western blotting.

## RESULTS

*Cholesterol Depletion Enhances MAP Kinase Activity and EGF Receptor Autophosphorylation.* Previous reports have shown that depletion of cholesterol from Rat-1 cells using methyl-$\beta$-cyclodextrin enhances EGF activation of MAP kinase (*10*). Similarly, treatment of NIH 3T3 cells with methyl-$\beta$-cyclodextrin led to an increase in the ability of EGF to stimulate MAP kinase activation with no change in the levels of MAP kinase itself (Figure 1A).

To determine whether changes in the EGF receptor itself contributed to the enhanced ability of EGF to stimulate MAP kinase activity in cholesterol-depleted cells, EGF receptor autophosphorylation was compared in control and cholesterol-depleted cells. As shown in Figure 1B, treatment with methyl-$\beta$-cyclodextrin led to a significant increase in the tyrosine phosphorylation of a protein in cell lysates with a molecular mass of ~170 kDa. This protein was identified as the EGF receptor since it could be specifically immunoprecipitated with anti-EGF receptor antibodies (right panel, Figure 1B). Maximal stimulation of receptor autophosphorylation by cholesterol depletion ranged from 2- to 5-fold in different experiments.

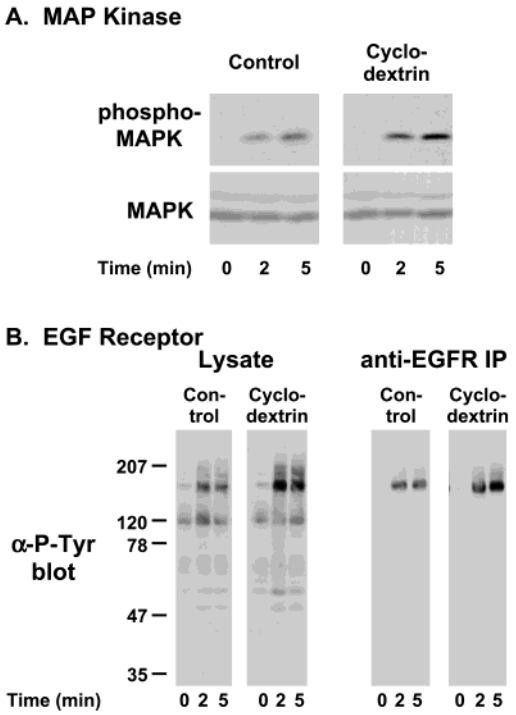


FIGURE 1: Effect of methyl-$\beta$-cyclodextrin on EGF receptor autophosphorylation. 3T3 cells were treated for 30 min at 37 °C with or without 7.5 mM methyl-$\beta$-cyclodextrin. Cells were subsequently stimulated with 25 nM EGF for 2 or 5 min and lysates prepared. Lysates (100 $\mu$g) either were applied directly to SDS−polyacrylamide gels or were immunoprecipitated (300 $\mu$g) with anti-EGF receptor antibodies before being analyzed by SDS−polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose and analyzed by Western blotting. (A) Western blot for phospho-MAP kinase and MAP kinase. (B) Western blot for anti-phosphotyrosine.

Figure 2 shows the effect of cholesterol depletion on the time course of EGF-stimulated receptor autophosphorylation in NIH 3T3 cells. In control cells, EGF-stimulated receptor autophosphorylation peaked 2 min after the addition of EGF and declined thereafter. A similar overall time course was observed in cyclodextrin-treated cells. However, EGF receptor autophosphorylation was higher at all time points in cholesterol-depleted cells as compared to control cells.

To determine whether the increase in EGF-stimulated receptor autophosphorylation was specifically due to the removal of cholesterol from the cell membranes, cholesterol was added back to control and cholesterol-depleted 3T3 cells using complexes of cholesterol and methyl-$\beta$-cyclodextrin. Treatment of 3T3 cells with methyl-$\beta$-cyclodextrin led to approximately a 40−60% decrease in the cellular cholesterol level (see Figure 3 legend). As shown in Figure 3, this depletion of cholesterol was associated with an increase in EGF-stimulated receptor autophosphorylation. This enhancement was progressively reversed by the addition of increasing amounts of cholesterol. At the highest concentration of cholesterol/methyl-$\beta$-cyclodextrin complexes, receptor autophosphorylation was inhibited by ~90% relative to that in the maximally cholesterol-depleted cells. These data indicate that the changes in EGF receptor autophosphorylation are the result of alterations in cholesterol levels rather than nonspecific effects of methyl-$\beta$-cyclodextrin.

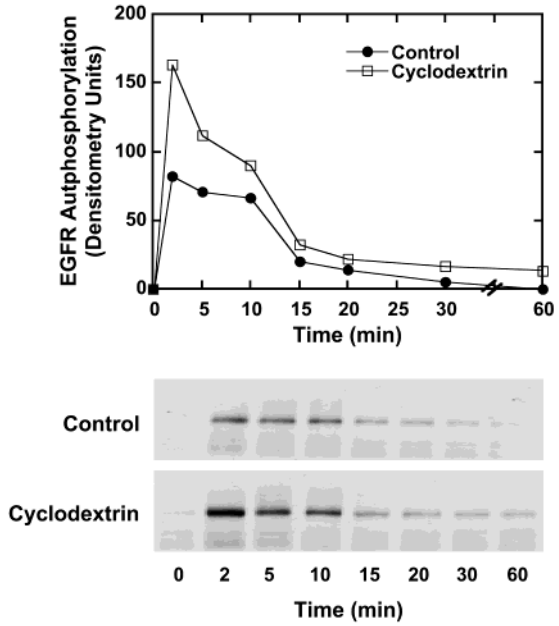


FIGURE 2: Time course of EGF receptor autophosphorylation in control and cholesterol-depleted cells. 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodextrin) as described under Experimental Procedures. The treatment medium was removed and replaced with DMEM containing 50 mM HEPES, pH 7.2, and 0.1% bovine serum albumin. Cells were stimulated with 25 nM EGF for the indicated times; lysates were prepared and analyzed for EGF receptor autophosphorylation by Western blotting with an anti-phosphotyrosine antibody. Receptor autophosphorylation was quantitated by densitometry. Upper panel: quantified results. Lower panel: Western blots of autophosphorylated EGF receptors.

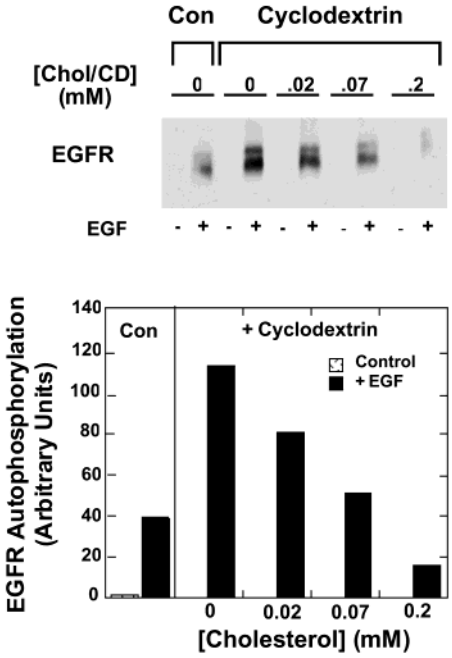


FIGURE 3: Repletion of cholesterol using cholesterol/methyl-$\beta$-cyclodextrin complexes. 3T3 cells were treated without or with 7.5 mM methyl-$\beta$-cyclodextrin as described under Experimental Procedures. After being washed, cultures were incubated with the indicated concentrations of cholesterol/methyl-$\beta$-cyclodextrin complexes where the concentration refers to the amount of cholesterol present. After 30 min, the medium was removed and replaced with DMEM containing 50 mM HEPES, pH 7.2, and 0.1% bovine serum albumin. Cells were stimulated for 5 min with 25 nM EGF, lysed, and analyzed for receptor autophosphorylation by Western blotting with an anti-phosphotyrosine antibody Upper panels: anti-phosphotyrosine Western blots of EGF receptors. Lower panels: quantitated results. Cellular cholesterol levels in 3T3 cells were the following: untreated cells, 21.2 $\pm$ 2.2 $\mu$g of cholesterol/mg of protein; cyclodextrin-treated cells, 7.0 $\pm$ 0.1 $\mu$g of cholesterol/mg of protein; and cholesterol-depleted cells repleted with 0.2 mM cholesterol/methyl-$\beta$-cyclodextrin, 30.3 $\pm$ 0.4 $\mu$g of cholesterol/mg of protein.

*Cholesterol Depletion Increases Cell Surface $^{125}$I-EGF Binding.* To identify the locus of the cholesterol-dependent alterations in EGF receptor autophosphorylation activity, the characteristics of $^{125}$I-EGF binding to control and cholesterol-depleted NIH 3T3 cells were assessed. Scatchard plots comparing $^{125}$I-EGF binding in these cells are shown in Figure 4. In both control and cyclodextrin-treated cells, the Scatchard plots were curvilinear, indicating the presence of two classes of EGF binding sites—one of high affinity ($\sim$20 pM) and one of low affinity ($\sim$2 nM). No significant change in the affinity of EGF for either of these sites was observed following treatment with methyl-$\beta$-cyclodextrin. However, depletion of cholesterol led to a modest increase in the number of EGF receptors (mean = 41%, $p < 0.01$, $n = 3$).

Two possible explanations could account for the changes in cell surface EGF binding associated with decreases in cholesterol content. First, depletion of cholesterol could affect the basal trafficking of the EGF receptor, leading to an accumulation of the receptor on the cell surface in cholesterol-depleted cells. Alternatively, changes in cholesterol content could alter the ability of the EGF receptor to access or bind its ligand, leading to only *apparent* changes in the number of cell surface receptors.

To distinguish between these two possibilities, sulfo-NHS S-S-biotin was used to label cell surface proteins following treatment of the cultures with methyl-$\beta$-cyclodextrin. Cell surface proteins were then isolated on streptavidin−agarose beads. Equal fractions of the starting cell lysate, the flow-through from the beads (containing intracellular proteins), and the eluate from the streptavidin−agarose beads (containing cell surface proteins) were analyzed for EGF receptor content by Western blotting. If externalization of an internal pool of EGF receptors were responsible for the increase in receptor number observed in methyl-$\beta$-cyclodextrin-treated

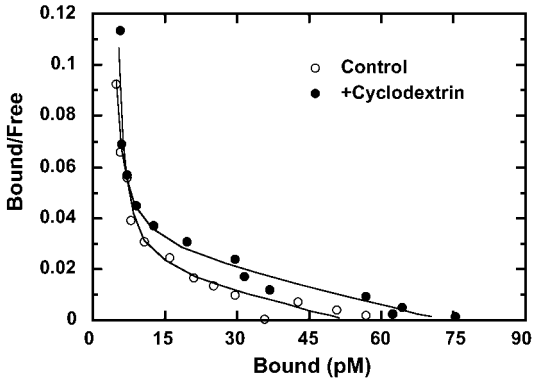


FIGURE 4: Scatchard plots of $^{125}$I-EGF binding to control and cholesterol-depleted cells. 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (+Cyclodextrin) as indicated under Experimental Procedures. The treatment medium was removed and replaced with DMEM containing 50 mM HEPES, pH 7.2, 0.1% bovine serum albumin, and $^{125}$I-EGF at varying concentrations. Binding was performed and quantitated as described under Experimental Procedures. All points represent the mean of triplicate determinations.

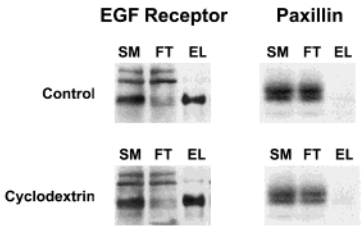


FIGURE 5: Cell surface biotinylation of control and cholesterol-depleted cells. 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodextrin) and subjected to cell surface biotinylation as described under Experimental Procedures. Equal aliquots (100 $\mu$L) of starting lysate (SM), the flow-through from the streptavidin−agarose columns containing internal receptors (FT), and the entire eluate from the streptavidin−agarose beads containing the cell surface receptors (derived from 100 $\mu$L of lysate) (EL) were analyzed by SDS−polyacrylamide gel electrophoresis and Western blotting with (left panel) an anti-EGF receptor antibody and (right panel) an anti-paxillin antibody.

cells, then the number of biotinylated EGF receptors should increase and the number of intracellular, nonbiotinylated receptors should decrease relative to control cells. However, if the changes in receptor number were due to alterations in the ability of the receptor to bind ligand, then no change in the relative number of cell surface and intracellular receptors would be expected in cholesterol-depleted cells.

The results of the cell surface biotinylation experiment are shown in Figure 5. The left panel shows the results for the EGF receptor while the right panel shows the results for paxillin, an intracellular protein used as a control. In both control and cyclodextrin-treated cells, paxillin was quantitatively recovered in the flow-through of the streptavidin−agarose column. This demonstrates that cell integrity was not disrupted by the treatment with methyl-$\beta$-cyclodextrin since this intracellular protein was not biotinylated.

By contrast, a significant fraction of the EGF receptor was biotinylated. When solubilized lysates from control and cyclodextrin-treated cells were incubated with streptavidin−agarose, only a relatively small proportion of the EGF receptor was present in the flow-through containing intracellular proteins. In four experiments, the average fraction of intracellular receptors was 18 $\pm$ 8% and 16 $\pm$ 6% of the total in lysates from control and cyclodextrin-treated cells, respectively. The fact that there was no difference in the amount of intracellular EGF receptor in control and cyclodextrin-treated cells suggests that cholesterol depletion does not induce the externalization of intracellular EGF receptors. Further, the size of the internal pool (~20%) appears to be less than the apparent increase in $^{125}$I-EGF binding (~40%), indicating that there is insufficient internal receptor to account for all of the observed increase in binding. Finally, no obvious increase in the level of (biotinylated) cell surface EGF receptors was noted in cyclodextrin-treated cells as compared to controls. Together these data suggest that little, if any, externalization of the EGF receptor occurred as a result of cholesterol depletion.

*Cholesterol Depletion Enhances EGF Receptor Kinase Activity.* While the increase in $^{125}$I-EGF binding observed following cholesterol depletion could contribute to the enhancement of EGF receptor autophosphorylation under these conditions, the size of the increase (~40%) does not appear sufficient to account for the 2−5-fold increase in receptor autophosphorylation. Thus, additional experiments

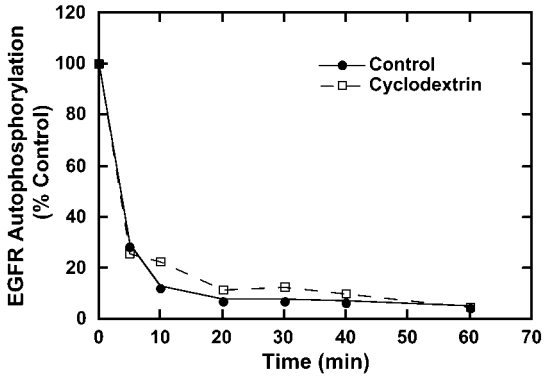


FIGURE 6: Dephosphorylation of the EGF receptor in control and cholesterol-depleted cells. 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodextrin) as outlined under Experimental Procedures. The treatment medium was removed and replaced with DMEM containing 50 mM HEPES, pH 7.2, and 0.1% bovine serum albumin. Cells were stimulated for 5 min with 25 nM EGF at 37 °C. Subsequently, the medium was removed, and the cells were washed twice in 50 mM glycine, 250 mM NaCl, pH 4, to remove surface-bound EGF. Warmed DMEM containing 50 mM HEPES, pH 7.2, and 0.1% bovine serum albumin was then added, and the cells were incubated at 37 °C for the indicated times. Cells were lysed and analyzed for EGF receptor autophosphorylation by Western blotting with an anti-phosphotyrosine antibody. Results were quantitated by densitometry.

were carried out to specifically assess the effects of methyl-$\beta$-cyclodextrin treatment on receptor kinase activity.

The increase in receptor autophosphorylation associated with cholesterol depletion could be due either to a decrease in phosphotyrosine phosphatase activity directed against the receptor or to an increase in intrinsic receptor kinase activity. To distinguish between these two possibilities, 3T3 cells were treated with or without methyl-$\beta$-cyclodextrin for 30 min and then with or without EGF for 5 min at 37 °C. After a wash with pH 4 glycine buffer to remove cell surface EGF, the cultures were incubated for increasing lengths of time at 37 °C in DMEM prior to lysis and analysis for EGF receptor autophosphorylation. As shown in Figure 6, autophosphorylation of the EGF receptor was rapidly reversed in control cells, returning to basal levels ~10 min after EGF withdrawal. Receptor dephosphorylation followed a similar rapid time course in methyl-$\beta$-cyclodextrin-treated cells. Thus, the increase in EGF receptor autophosphorylation observed in cholesterol-depleted cells does not appear to be due to a decrease in the activity of a phosphatase that dephosphorylates EGF receptor.

To investigate the possibility that intrinsic receptor tyrosine kinase activity was enhanced by cholesterol depletion, 3T3 cells were treated in the absence or presence of methyl-$\beta$-cyclodextrin for 30 min, and then a total membrane fraction was prepared. These membranes were then treated with EGF and assayed in an in vitro kinase assay that contained protease inhibitors as well as numerous phosphatase inhibitors. The results, shown in Figure 7A, demonstrate that EGF-stimulated receptor autophosphorylation was enhanced in membranes prepared from cells that had been depleted of cholesterol as compared to control cells despite equal receptor loading. The average increase in receptor autophosphorylation following cholesterol depletion was (6.1 $\pm$ 2.8)-fold ($n$ = 3). Similarly, when these membranes were assayed in vitro using an exogenous tyrosine-containing peptide as substrate (Figure 7B), the EGF-stimulated tyrosine kinase activity was

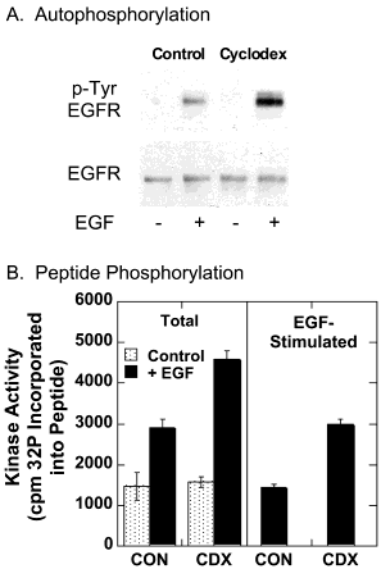


FIGURE 7: In vitro kinase activity of EGF receptors derived from control and cholesterol-depleted cells. 3T3 cells were treated without (CON) or with 7.5 mM methyl-$\beta$-cyclodextrin (CDX), and membranes were prepared. Aliquots of membranes were subjected to in vitro kinase assays as outlined under Experimental Procedures. (A) Anti-phosphotyrosine and anti-EGF receptor Western blots of membranes derived from control and cyclodextrin-treated cells. (B) Kinase activity of the EGF receptor present in membranes derived from control and cyclodextrin-treated cells measured using an exogenous peptide substrate, Arg-Arg-Src.

significantly greater in membranes derived from cholesterol-depleted cells as compared to control cells. The average fold increase was (2.0 $\pm$ 0.2)-fold ($n$ = 4). Thus, cholesterol depletion appears to increase the intrinsic tyrosine kinase activity of the EGF receptor.

*Cholesterol Depletion Alters the Partitioning of EGF Receptors into Lipid Rafts.* Cholesterol depletion has been shown to promote the loss of EGF receptors from cholesterol-enriched lipid raft domains (*4*). To determine whether the subcellular localization of the EGF receptor was altered in the cells that had been treated with methyl-$\beta$-cyclodextrin, lipid rafts were prepared from control and cholesterol-depleted cells, and the ability of the EGF receptor to partition into low-density membrane domains was assessed. As shown in Figure 8A, a large portion of the lipid raft marker protein flotillin (*19*) was found in the low-density portion of the gradient. Depletion of cholesterol with methyl-$\beta$-cyclodextrin lead to the loss of flotillin from the low-density fraction and its recovery in the higher density region of the gradient. Similarly, the EGF receptor (Figure 8B) was found in the low-density fractions of the gradient in the control cells but was lost from this region in cells depleted of cholesterol. These data suggest that treatment with methyl-$\beta$-cyclodextrin results in the disruption of low density, cholesterol-enriched domains and the loss of the EGF receptor from this compartment.

*Cholesterol Depletion Impairs Internalization and Down-regulation of EGF*. The magnitude of EGF-stimulated signaling is modulated not only by the level of the initial signal but also by the rate at which the EGF receptor is internalized and down-regulated. To determine whether these aspects of EGF receptor function were affected by cholesterol depletion, 3T3 cells were treated with or without methyl-$\beta$-cyclodextrin, and their ability to internalize $^{125}$I-EGF was

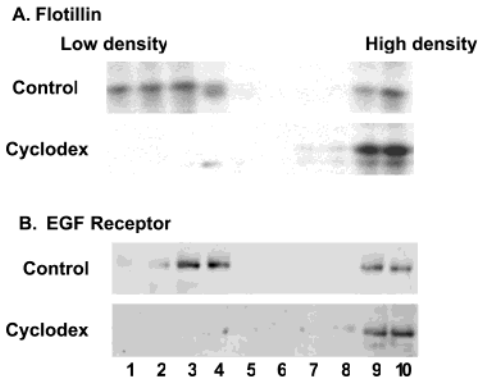


FIGURE 8: Distribution of EGF receptors in lipid rafts prepared from control and cholesterol-depleted cells. 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodex). Lipid rafts were prepared as outlined under Experimental Procedures. Equal aliquots of fractions from the Opti-Prep gradient were run on an SDS gel and Western-blotted for flotillin (panel A) or EGF receptors (panel B).

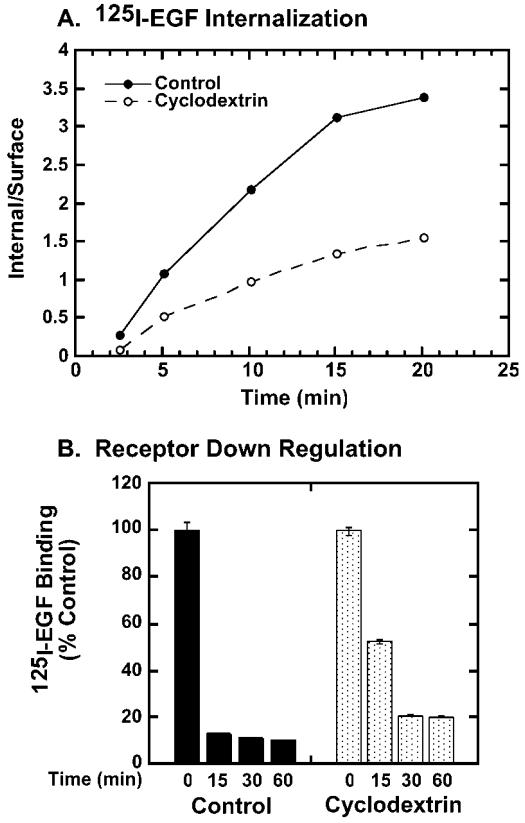


FIGURE 9: Internalization and down-regulation of EGF receptors in control and cholesterol-depleted cells. (Panel A) 3T3 cells were treated without medium (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodextrin). Subsequently, the cells were incubated with 2 nM $^{125}$I-EGF for the indicated times at 37 °C. Internalization of the bound $^{125}$I-EGF was measured as described under Experimental Procedures. (Panel B) 3T3 cells were treated without (Control) or with 7.5 mM methyl-$\beta$-cyclodextrin (Cyclodextrin), washed, and incubated with 25 nM EGF for 30 min at 37 °C. After being washed with low-pH buffer to remove residual surface-bound EGF, cells were incubated with 2 nM $^{125}$I-EGF for 2 h at 4 °C, and binding measured as described under Experimental Procedures.

assessed. As shown in Figure 9A, cells depleted of cholesterol exhibited a significantly slower rate of internalization of the $^{125}$I-EGF than control cells.

To determine whether the cholesterol-induced alterations in receptor internalization led to changes in receptor down-

regulation, the ability of EGF to promote a reduction in cell surface EGF receptors was assessed in control and cholesterol-depleted cells. 3T3 cells were treated with or without cyclodextrin and then incubated in the absence or presence of 25 nM EGF for increasing times at 37 °C to induce receptor down-regulation. Following a low-pH wash to remove residual cell surface EGF, the binding of $^{125}$I-EGF to the cells was assessed. As shown in Figure 9B, EGF receptors in control cells were rapidly down-regulated following agonist treatment. As early as 15 min after EGF addition, approximately 90% of the receptors had been removed from the cell surface. Receptor down-regulation was significantly slower in cyclodextrin-treated cells. After 15 min with EGF, cholesterol-depleted cells showed only about a 40−50% loss of agonist binding. These data are consistent with the pattern of alterations in $^{125}$I-EGF internalization shown in Figure 9A and indicate that cholesterol depletion impairs EGF-stimulated receptor internalization and down-regulation.

## DISCUSSION

In this report, we show that the function and trafficking of the EGF receptor are modulated by cholesterol depletion in ways that would serve to enhance EGF receptor signaling. Depletion of cholesterol was associated with an ∼40% increase in cell surface $^{125}$I-EGF binding. Cell surface biotinylation studies indicated that there was no significant decrease in the number of intracellular receptors in cholesterol-depleted cells nor increase in the number of cell surface receptors. Thus, depletion of cholesterol seems to facilitate the binding of EGF to preexisting cell surface receptors. Even if some externalization did occur, the pool of intracellular EGF receptors (∼15−20% of total) is insufficient to account for the magnitude of the increase in EGF binding to methyl-$\beta$-cyclodextrin-treated cells (∼40%). Consistent with our findings, Roepstorff et al. (*20*) used ATP depletion to conclude that no receptor externalization occurred in cyclodextrin-treated HeLa cells. By contrast, Ringerike et al. (*21*) concluded that the enhanced EGF binding seen in A431 and HepG2 cells following cholesterol depletion was due to externalization of EGF receptors from intracellular pools. However, the fraction of the receptor in intracellular pools was not reported in that study so it is difficult to determine whether sufficient internal receptors were available to account for the increase in cell surface binding. It is possible that the effect of cholesterol depletion on receptor distribution differs in different cell types.

Depletion of cholesterol from 3T3 cells enhanced in vivo EGF-stimulated receptor autophosphorylation by 100−400% without affecting basal levels of receptor phosphorylation. This differs from the observations of Ringerike et al. (*21*), who found that cholesterol depletion enhanced both basal and stimulated EGF receptor phosphorylation in A431 cells. These discrepancies may be due to the different cell types used or to the different level of expression of EGF receptors in these two cell types.

While the apparent ∼40% increase in cell surface EGF receptors could contribute to the observed 2−5-fold increase in EGF receptor autophosphorylation, the magnitude of the increase in receptor number appears to be too low to account for the entire increase observed in receptor autophosphorylation. No change in the rate of receptor dephosphorylation was observed after cholesterol depletion. However, in vitro kinase assays demonstrated at least a 2-fold increase in receptor tyrosine kinase activity as measured by receptor autophosphorylation and phosphorylation of an exogenous tyrosine-containing peptide. As these assays are done under conditions in which phosphotyrosine phosphatases are inhibited and receptor trafficking cannot occur, these findings suggest that cholesterol depletion leads to an increase in the intrinsic tyrosine kinase activity of the EGF receptor.

EGF receptors have been shown to selectively partition into caveolae and other cholesterol-enriched membrane domains (*2*−*4*), and we show here that cholesterol depletion results in the redistribution of the EGF receptor out of this compartment. The apparent increase in EGF receptor binding and intrinsic kinase activity associated with the movement of the EGF receptor out of lipid rafts is consistent with the interpretation that EGF receptor function is normally somewhat suppressed when it is in this compartment. Cholesterol depletion may relieve this inhibition by disrupting lipid rafts, allowing exit of the EGF receptor from these domains.

Exactly how cholesterol or lipid raft association modulates EGF receptor function is not clear. It is possible that the cholesterol-associated changes in the EGF receptor may be due, at least in part, to changes in the structure or function of lipid rafts. Cholesterol is known to affect the packing of lipids in bilayers, and it is possible that cholesterol alters EGF receptor function by changing lipid packing and inducing conformational changes in the receptor itself. Alternatively, other lipid or protein components of lipid rafts/caveolae could exercise an inhibitory effect on the EGF receptor that is relieved when the domains are disrupted by cholesterol. Caveolin-1 is a potential candidate for such a role. However, we have observed that the enhancement of EGF receptor autophosphorylation upon cholesterol depletion occurs even in cells that lack caveolin.[2] In addition, the recent work of Ringerike et al. (*21*) suggests that few EGF receptors are found in caveolae. Thus, if cholesterol suppresses receptor function by promoting its incorporation into rafts, it seems unlikely that caveolin-1 is responsible for the inhibition of receptor kinase activity in this compartment. Ganglioside GM3, a component of lipid rafts, has been reported to inhibit EGF-mediated signaling (*22*, *23*). Relief from inhibition by this compound upon release of the receptor from lipid rafts could contribute to the observed increase in tyrosine kinase activity.

Treatment with methyl-$\beta$-cyclodextrin resulted in the inhibition of $^{125}$I-EGF internalization. This observation is consistent with previous reports that cholesterol depletion blocks the budding of coated pits and reduces endocytosis of EGF (*24*, *25*). This impairment of internalization led to a decrease in the rate at which the EGF receptor was cleared from the cell surface in the process of down-regulation. In combination with the observed increases in EGF binding and receptor tyrosine kinase activity, this change in trafficking could serve to enhance signaling downstream of the EGF receptor. Cholesterol depletion has been reported to enhance EGF-stimulated MAP kinase activation (*10*), and we have also observed this effect in 3T3 cells in this study. Our data

[2] Pike, L. J., and MacDonald, J., unpublished results.

suggest that at least a portion of the increase in MAP kinase activation seen in cholesterol-depleted cells is likely to be due to effects on both EGF receptor function and trafficking.

Our findings suggest that the function of the EGF receptor itself is affected by cholesterol depletion, possibly due to release of the receptor from inhibitory constraints placed on its activity by localization to lipid rafts. The fact that cholesterol depletion enhances EGF receptor kinase activity yet impairs EGF-stimulated phosphatidylinositol turnover (*4*) suggests that the role of cholesterol and lipid rafts in supporting cell signaling is complex and involves effects on multiple components of the pathways. The data presented here begin to identify the molecular basis by which cholesterol levels modulate cell signaling.

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