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# Cholesterol Depletion Results in Site-specific Increases in Epidermal Growth Factor Receptor Phosphorylation due to Membrane Level Effects

STUDIES WITH CHOLESTEROL ENANTIOMERS*

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**In A431 cells, depletion of cholesterol with methyl-β-cyclodextrin induced an increase in both basal and epidermal growth factor (EGF)-stimulated EGF receptor phosphorylation. This increase in phosphorylation was site-specific, with significant increases occurring at $Tyr^{845}$, $Tyr^{992}$, and $Tyr^{1173}$, but only minor changes at $Tyr^{1045}$ and $Tyr^{1068}$. The elevated level of receptor phosphorylation was associated with an increase in the intrinsic kinase activity of the EGF receptor kinase, possibly as a result of the cyclodextrin-induced enhancement of the phosphorylation of $Tyr^{845}$, a site in the kinase activation loop known to be phosphorylated by $pp60^{src}$. Cholesterol and its enantiomer (*ent*-cholesterol) were used to investigate the molecular basis for the modulation of EGF receptor function by cholesterol. Natural cholesterol (*nat*-cholesterol) was oxidized substantially more rapidly than *ent*-cholesterol by cholesterol oxidase, a protein that contains a specific binding site for the sterol. By contrast, the ability of *nat*- and *ent*-cholesterol to interact with sphingomyelins and phosphatidylcholine and to induce lipid condensation in a monolayer system was the same. These data suggest that, whereas cholesterol-protein interactions may be sensitive to the absolute configuration of the sterol, sterol-lipid interactions are not. *nat*- and *ent*-cholesterol were tested for their ability to physically reconstitute lipid rafts following depletion of cholesterol. *nat*- and *ent*-cholesterol reversed to the same extent the enhanced phosphorylation of the EGF receptor that occurred following removal of cholesterol. Furthermore, the enantiomers showed similar abilities to reconstitute lipid rafts in cyclodextrin-treated cells. These data suggest that cholesterol most likely affects EGF receptor function because of its physical effects on membrane properties, not through direct enantioselective interactions with the receptor.**

Cholesterol is an essential component of mammalian membranes. It alters membrane fluidity, thickness, curvature, and permeability (1–6). In addition, cholesterol is an important constituent of lipid rafts, specialized membrane microdomains that are rich in cholesterol, sphingolipids, and saturated phospholipids (1, 6, 7). Through interactions with cholesterol, the acyl chains of phospholipids in lipid rafts pack tightly together and extend fully to create a liquid-ordered phase (7–9).

A subset of plasma membrane proteins selectively partition into the ordered environment of lipid rafts (1, 7, 10). Because of the large number of signaling proteins that are localized to lipid rafts, it has been postulated that these domains serve as regulatory platforms for some signal transduction pathways (11, 12). The epidermal growth factor (EGF)[1] receptor is one of the proteins involved in signaling that is known to be enriched in lipid rafts (13–15).

A variety of studies have shown that EGF receptor function is affected by the levels of cholesterol, which is present at higher concentrations in lipid rafts than in the surrounding plasma membrane (16). Depletion of cholesterol from cells leads to an increase in both basal (17, 18) and EGF-stimulated (18, 19) receptor phosphorylation. The enhanced receptor tyrosine phosphorylation appears to be due to a rise in the intrinsic kinase activity of the receptor (19). Cholesterol depletion has also been shown to result in an increase in the number of cell-surface EGF-binding sites (19, 20) The increased EGF binding seems to result from an unmasking of receptors that are present on the surface of cells, but are unable to bind EGF in the presence of elevated levels of cholesterol (19, 20).

In addition to altering the intrinsic binding and kinase activity of the EGF receptor, cholesterol also modulates signaling events directly downstream of the EGF receptor. For example, depletion of cholesterol impairs the ability of EGF to stimulate phosphatidylinositol turnover (21). By contrast, cholesterol depletion leads to the enhancement of EGF-stimulated MAPK activity (22).

These observations suggest that cholesterol plays a significant role in modulating EGF receptor-mediated signaling. However, the molecular basis for these effects of cholesterol on EGF receptor function is not known. Cholesterol could affect EGF receptor function indirectly by influencing the physical

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[1] The abbreviations used are: EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; DMEM, Dulbecco's modified Eagle's medium; *nat*-cholesterol, natural cholesterol; *ent*-cholesterol, enantiomer of cholesterol; PBS, phosphate-buffered saline; BSA, bovine serum albumin; MES, 4-morpholineethanesulfonic acid.

properties of the membrane, such as thickness, fluidity, or lateral domain formation (3, 23). Indeed, depletion of cholesterol leads to the loss of the EGF receptor from low density lipid raft domains (19, 21), suggesting that some of the effects of cholesterol on EGF receptor function could be mediated through the ability of the sterol to promote lateral membrane domain formation. Alternatively, cholesterol could bind specifically to the receptor, regulating its activity through allosteric mechanisms. In this study, we further characterize the effects of cholesterol depletion on EGF receptor function and address the question of whether the effects of cholesterol are due to membrane level effects of the compound or result from specific molecular recognition of the sterol.

We report here that the enhanced basal and hormone-stimulated phosphorylation of the EGF receptor that occurs upon cellular cholesterol depletion is due to a selective increase in the phosphorylation of a subset of the phosphorylatable tyrosine residues in the C-terminal tail of the receptor. Using natural cholesterol (*nat*-cholesterol) and its enantiomer (*ent*-cholesterol), we provide evidence that the effects of cholesterol on EGF receptor function are most likely due to non-enantioselective effects of the sterol on membrane properties such as fluidity and the ability to form rafts.

## EXPERIMENTAL PROCEDURES

### *Materials*

EGF was prepared by the method of Savage and Cohen (24). The anti-phosphotyrosine monoclonal antibody PY20 was from Transduction Laboratories (Lexington, KY). Polyclonal antibodies against $Tyr(P)^{845}$, $Tyr(P)^{992}$, $Tyr(P)^{1045}$, and $Tyr(P)^{1068}$ of the EGF receptor were purchased from Cell Signaling Technology (Beverly, MA). A monoclonal antibody against $Tyr(P)^{1173}$ of the EGF receptor was from Upstate Biotechnology, Inc. (Lake Placid, NY). The anti-flotillin monoclonal antibody was from BD Transduction Laboratories (San Diego, CA). Polyvinylidene fluoride membranes were from Osmonics, Inc. (Westborough, MA). The enhanced chemiluminescence kit was from Amersham Biosciences. The cholesterol CII and free cholesterol E assay kits were from Wako Bioproducts (Richmond, VA). Methyl-β-cyclodextrin was from Aldrich. Egg sphingomyelin and (2*S*,3*R*,4*E*)-2-stearoylaminooctadec-4-ene-3-hydroxy-1-phosphocholine (*N*-stearoylsphingomyelin) were from Avanti Polar Lipids (Alabaster, AL). All other chemicals were from Sigma.

### *Methods*

*Cell Culture*—A431 cells were maintained at 37 °C and 5% $CO_2$ in Dulbecco's modified Eagle's medium (DMEM) containing 7% newborn calf serum and 3% fetal calf serum. Cells were incubated overnight in DMEM containing 0.1% newborn calf serum prior to use. Chinese hamster ovary cells were maintained at 37 °C and 5% $CO_2$ in Ham's F-12 medium containing 10% fetal calf serum. Cells were incubated overnight in Ham's F-12 medium containing 0.1% fetal calf serum prior to use.

*Synthesis of ent-Cholesterol*—*ent*-Cholesterol was prepared from *ent*-desmosterol through a minor modification of methods described previously (25). Silyl-protected *ent*-desmosterol was subjected to catalytic hydrogenation (300 p.s.i. $H_2$, 15 min, platinum/carbon) and then treated to remove the silyl protecting group to give *ent*-cholesterol (m.p. 147.5–148 °C, $[\alpha]_D^{20} = +40.0$ C (*C*, 1.0, in $CHCl_3$)). Fig. 1 shows the structures of *nat*- and *ent*-cholesterol.

*Preparation of Cholesterol·Methyl-β-cyclodextrin Complexes*—Cholesterol·methyl-β-cyclodextrin complexes were synthesized as described by Klein *et al.* (26). Briefly, 30 mg of *nat*- or *ent*-cholesterol was dissolved in 400 μl of isopropyl alcohol/chloroform (2:1, v/v). Methyl-β-cyclodextrin (1 g) was dissolved in 11 ml of phosphate-buffered saline (PBS) and heated to 80 °C with stirring. The solubilized sterol was added in small aliquots to the heated solution over 30 min.

*Cholesterol Depletion and Repletion*—For cholesterol depletion, cells were incubated for 30 min at 37 °C in DMEM containing 50 mM HEPES (pH 7.2) and 0.1% bovine serum albumin (DMEM/BSA) and the indicated concentration of methyl-β-cyclodextrin. Control cells were incubated in the same medium lacking cyclodextrin. To replete cells with cholesterol following cyclodextrin treatment, cells were incubated at 37 °C for 30 min in DMEM/BSA containing the indicated concentration of sterol·cyclodextrin complex. For both cholesterol depletion and repletion, the tissue culture plates were swirled every 10 min to ensure continuous mixing of the components in the medium.

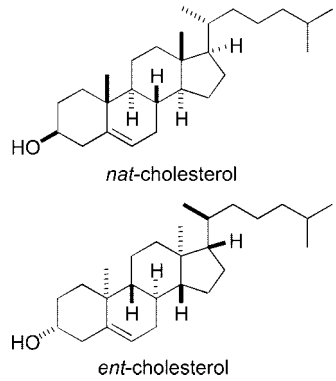


FIG. 1. **Structures of *nat*-cholesterol and its unnatural enantiomer, *ent*-cholesterol.**

*Cholesterol Assay*—Cells were washed twice with 1 ml of cold PBS, and lipids were extracted with 2 ml of hexane/isopropyl alcohol (3:2, v/v) for 1 h at room temperature. The organic extract was removed from the cell monolayer, and the solvent was removed in a SpeedVac. The lipid residue was solubilized in 1 ml of the cholesterol CII assay kit buffer solution. As reported previously (27), color generation from *nat*-cholesterol standards reached a plateau after 5 min at 37 °C and remained stable through 60 min at this temperature, whereas *ent*-cholesterol standards required 60 min at 37 °C for complete color development. Samples were incubated for 1 h at 37 °C prior to measuring absorbance at 505 nm.

After lipid extraction, the residual cell monolayers were solubilized with 10 mM sodium borate and 1% SDS. Aliquots of the solubilized material were then used for determination of total protein content using the bicinchoninic acid protein assay.

*Cholesterol Oxidase Assay*—Assay of cholesterol oxidase was carried out utilizing the free cholesterol E assay kit. The free cholesterol E assay reagent contains cholesterol oxidase plus several additional components (peroxidase, 4-aminoantipyrene, and DEHSA (3-((3,5-dimelhoxyphenyl)ethylamino)-2-hydroxy-1-propanoic acid monosodium salt)) that permit the colorimetric detection of the hydrogen peroxide produced upon oxidation of cholesterol. For assay, 5 μg of either *nat*- or *ent*-cholesterol in complex with cyclodextrin was placed in a glass tube. One ml of the free cholesterol E assay kit buffer solution was then added. Samples were incubated at 37 °C for the indicated times. The absorbance of the samples at 600 nm was measured immediately. Equal concentrations of free cholesterol and cyclodextrin-complexed cholesterol gave equivalent absorbance measurements in this assay.

*Cell-surface EGF Binding*—A431 cells were grown to confluence in 24-well dishes and incubated overnight in DMEM containing 0.1% fetal calf serum. After treatment to alter cholesterol content, cultures were washed with ice-cold PBS and incubated for 2 h at 4 °C in 1 ml of DMEM/BSA, 50 pM $^{125}$I-EGF, and increasing concentrations of unlabeled EGF. At the end of the incubation, cells were washed three times with ice-cold PBS. Cell monolayers were dissolved in 1 ml of 1 M NaOH and counted for $^{125}$I. Data were analyzed using the LIGAND computer program (28).

*Stimulation of Cells with EGF and Preparation of Cell Lysates*—Cells in 35-mm dishes were treated to alter cholesterol content as outlined above. At the end of the incubation, EGF was added to the medium for 5 min. Cells were then washed with cold PBS and lysed by scraping the monolayers into 300 μl of radioimmune precipitation assay buffer (10 mM Tris (pH 7.2), 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) containing 1 μg/ml leupeptin, 100 μM sodium *o*-vanadate, 10 mM *p*-nitrophenyl phosphate, and 1 mM phenylmethylsulfonyl fluoride. Lysates were incubated on ice for 10 min with periodic vortexing and then clarified by centrifugation at 12000 × *g* for 10 min. Aliquots were then taken for determination of total protein concentration. Receptor tyrosine phosphorylation was determined by Western blotting.

*EGF Receptor Dephosphorylation*—To assess the rate of EGF receptor dephosphorylation, cholesterol-depleted or cholesterol-repleted cells were stimulated with 1.25 nM EGF for 5 min at 37 °C. The medium was removed, and cells were washed with cold PBS. Residual cell surface-bound EGF was removed by incubating the cells twice for 2 min in 50 mM glycine and 100 mM NaCl (pH 4.0) on ice. After an additional wash

with cold PBS, warmed DMEM/BSA was added, and the cells were incubated at 37 °C for the indicated times. Cells were washed with cold PBS and lysed with radioimmune precipitation assay buffer as usual. Receptor tyrosine phosphorylation was determined by Western blotting.

*Membrane Preparation and in Vitro Phosphorylation Assays*—Cells were treated to alter cholesterol levels and then lysed by homogenization in 25 mM HEPES (pH 7.2). Membranes were pelleted by centrifugation for 10 min at 12000 × *g* and resuspended in 70 mM β-glycerophosphate, 250 mM NaCl, and 25% glycerol (pH 7.2). Assays were carried out in a final volume of 50 μl containing 20 mM β-glycerophosphate, 100 μM ATP, 12 mM $MgCl_2$, 2 mM $MnCl_2$, 20 mM *p*-nitrophenyl phosphate, 100 μM sodium *o*-vanadate, and 1 μg of membrane protein. When included, EGF was added at a final concentration of 25 nM. Membranes were incubated with growth factor for 5 min at room temperature. Assays were begun by the addition of ATP and metal ions. After incubation at 30 °C for 15 s, reactions were stopped by the addition of 50 μl of SDS sample buffer. Samples were boiled, run on a 10% SDS-polyacrylamide gel, and analyzed by Western blotting.

*Preparation of Lipid Rafts*—All manipulations were performed at 4 °C. After appropriate treatments, one 150-mm plate of A431 cells was washed five times with PBS and scraped into 0.4 ml of 0.25 M sucrose, 1 mM EDTA, and 20 mM Tris (pH 7.8). Cells were lysed by passage through a 23-gauge needle 10 times. The lysates were sonicated five times for 15 s using a Branson 250 sonicator set at maximal power output for a microtip. After centrifugation at 1000 × *g* for 10 min, the post-nuclear supernatant was mixed with an equal volume of 85% sucrose in MES-buffered saline (25 mM MES (pH 6.5), 150 mM NaCl, and 2 mM EDTA) and placed in the bottom of a centrifuge tube. A 15–35% discontinuous sucrose gradient was formed above the lysate by adding sucrose-containing buffers as follows: 2 ml of 35% sucrose, 2 ml of 28% sucrose, 2 ml of 22% sucrose, and 4 ml of 15% sucrose, all in MES-buffered saline. The gradient was centrifuged for 18 h at 210,000 × *g* in an SW 41 rotor (Beckman Instruments). After discarding the uppermost 4 ml, the gradient was fractionated into eight 1-ml fractions. Then, 100-μl aliquots of each fraction were subjected to SDS-PAGE and analyzed by Western blotting.

*Western Blotting*—Samples containing 25–100 μg of protein mixed with SDS sample buffer were subjected to SDS-PAGE and transferred electrophoretically to polyvinylidene fluoride membranes. The membranes were blocked with 10% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 and incubated with primary antibody for 1 h at room temperature (PY20, $Tyr(P)^{1173}$, DB-1, flotillin) or overnight at 4 °C ($Tyr(P)^{845}$, $Tyr(P)^{992}$, $Tyr(P)^{1045}$, and $Tyr(P)^{1068}$). Antibody detection was carried out using enhanced chemiluminescence.

*Langmuir Film Balance*—Water for the subphase buffer was purified by reverse osmosis, activated charcoal adsorption, and mixed-bed deionization; passed through a Milli-Q UV Plus system (Millipore Corp., Bedford, MA); and filtered through a 0.22-μm Millipak 40 membrane. Subphase buffer (pH 6.6) consisting of 10 mM potassium phosphate, 100 mM NaCl, and 0.2% sodium azide was stored under argon until used. Glassware was acid-cleaned and rinsed thoroughly with deionized water and then with hexane/ethanol (95:5). Solvent purity was verified by dipole potential measurements prior to use (29). Final stock concentrations of sterols were determined gravimetrically using a Cahn microbalance (Model 4700), and those of sphingomyelins by lipid phosphate analysis (30).

Surface pressure-molecular area isotherms were measured using a computer-controlled, Langmuir-type balance, described in detail previously (31, 32) and calibrated according to the equilibrium spreading pressures of known lipid standards (33). The subphase was maintained at a fixed temperature using a thermostatted circulating water bath. The film balance was housed in an isolated laboratory supplied with clean air by a Bioclean air filtration system equipped with charcoal and HEPA filters. The trough was separately enclosed under humidified argon and cleaned by passage through a seven-stage series filtration setup consisting of an Alltech activated charcoal gas purifier, a Lab-Clean filter, and a series of Balston disposable filters consisting of two adsorption (carbon) and three filter units (93 and 99.99% efficiency at 1 μm). Film balance features that contribute to isotherm reproducibility include automated lipid spreading via a modified high pressure liquid chromatography autoinjector, automated surface cleaning by multiple barrier sweeps between runs, and highly accurate reproducible setting of the subphase level by an automated aspirator. Lipids were mixed and spread (51.67-μl aliquots) from stock solutions dissolved in hexane/ethanol (95:5). Films were compressed at a rate of ≤4 $Å^2$/molecule/min after an initial delay period of 4 min. Standard errors of the resulting force-area isotherms were routinely <2%.

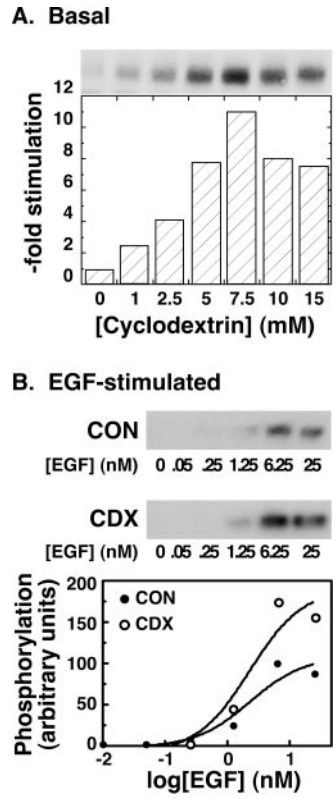


FIG. 2. **Effect of cholesterol depletion on EGF receptor phosphorylation.** *A*, A431 cells were treated without or with the indicated concentrations of methyl-β-cyclodextrin for 30 min to remove cholesterol. Cells were lysed and analyzed for basal receptor phosphorylation by Western blotting with anti-phosphotyrosine antibody PY20. Results were quantitated by densitometry. *B*, cells were treated without (control (*CON*)) or with 7.5 mM methyl-β-cyclodextrin (*CDX*) for 30 min and were then stimulated with the indicated doses of EGF for 5 min. Cells were lysed and analyzed as described for *A*. Results from a representative experiment were quantified by densitometry and subjected to nonlinear curve fitting using GraphPAD Prism.

## RESULTS

*Site-specific Effect of Cholesterol on EGF Receptor Phosphorylation*—A431 cells were treated with increasing concentrations of methyl-β-cyclodextrin for 30 min to remove cholesterol. Lysates were prepared and analyzed for receptor tyrosine phosphorylation by SDS-PAGE and Western blotting with an anti-phosphotyrosine antibody. As shown in Fig. 2*A*, cholesterol depletion increased basal tyrosine phosphorylation of the EGF receptor up to 11-fold. The dose of methyl-β-cyclodextrin yielding maximal stimulation was 7.5 mM when confluent cultures were used. However, when significantly subconfluent cells were used, the optimal concentration of methyl-β-cyclodextrin was 2–3-fold less than this. This indicates that it is the ratio of cells to reagent that is important rather than the absolute concentration of reagent added.

In addition to enhancing basal EGF receptor phosphorylation, cholesterol depletion also increased EGF-stimulated receptor phosphorylation at all doses of EGF tested (Fig. 2*B*). At the maximal dose of EGF, receptor phosphorylation was enhanced from 1.2- to 2.0-fold in different experiments. The $EC_{50}$ for EGF was ~2 nM and was essentially unchanged following cholesterol depletion. Thus, the increase in EGF-stimulated tyrosine phosphorylation of the receptor following cholesterol depletion appears to be due to a change in the maximal level of phosphorylation rather than to a change in the $EC_{50}$ for EGF.

The increase in EGF receptor phosphorylation was associated with only a modest increase in the number of cell-surface EGF-binding sites (Fig. 3). Scatchard analysis indicated the

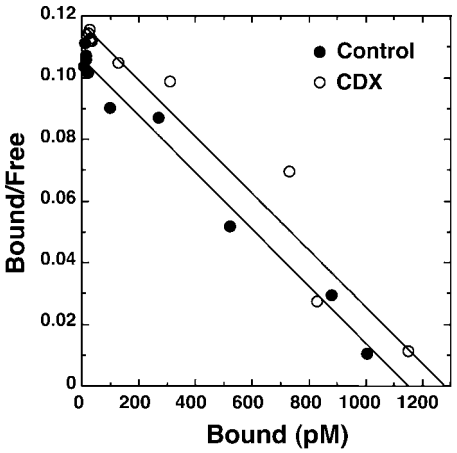


FIG. 3. **Effect of cholesterol depletion on $^{125}$I-EGF binding.** A431 cells were treated without (●) or with (○) 5 mM methyl-β-cyclodextrin (*CDX*) and assayed for $^{125}$I-EGF binding as described under "Experimental Procedures." Points represent the mean of triplicate determinations.

presence of a single class of EGF-binding sites on control cells that exhibited a $K_d$ of ~10 nM. Cyclodextrin treatment resulted in an ~10% increase in the number of EGF-binding sites present on A431 cells with no change in the binding affinity of EGF.

The EGF receptor contains several tyrosine residues that become phosphorylated in response to EGF. To determine whether all sites were similarly affected by cholesterol depletion, EGF receptor phosphorylation was analyzed using a panel of antibodies that recognize specific phosphorylated tyrosine residues on the receptor. The data in Fig. 4 show that cholesterol depletion differentially affected the phosphorylation of individual tyrosine residues. Cholesterol depletion routinely enhanced the EGF-stimulated phosphorylation of tyrosines 845, 992, and 1173. By contrast, the level of hormone-stimulated phosphorylation of tyrosines 1045 and 1068 was relatively unaffected by removal of cholesterol. In all cases, cholesterol depletion did not significantly alter the $EC_{50}$ for receptor phosphorylation. These data indicate that cholesterol depletion induces site-specific changes in phosphorylation of the EGF receptor.

*Comparison of the Enantiomers of Cholesterol*—The effect of cholesterol depletion on EGF receptor phosphorylation could be due either to an effect of cholesterol on the physical properties of the membrane or to direct interaction of the sterol with the EGF receptor or another protein that regulates receptor phosphorylation. Theoretically, the use of cholesterol enantiomers should allow discrimination between these two possibilities. Because the physical properties of *nat*-cholesterol and its enantiomer, *ent*-cholesterol, are identical, their effects on general membrane properties should not be significantly different. However, because the two enantiomers have mirror image shapes, they should interact differently with molecules, such as proteins, that contain a stereospecific binding site for the sterol. To determine whether cholesterol affects EGF receptor function due to general membrane level effects or to direct binding to a protein, the enantiomer of cholesterol was synthesized (25) and used as a tool to probe the effects of cholesterol on EGF receptor function.

The ability of *nat*- and *ent*-cholesterol to interact with lipids and proteins was first compared in model systems to characterize the behavior of these two enantiomers in sterol-protein and sterol-lipid interactions. To assess the characteristics of *nat*- and *ent*-cholesterol when interacting specifically with a protein, the ability of cholesterol oxidase to use these enantiomers as substrates was compared. As shown in Fig. 5, *nat*-

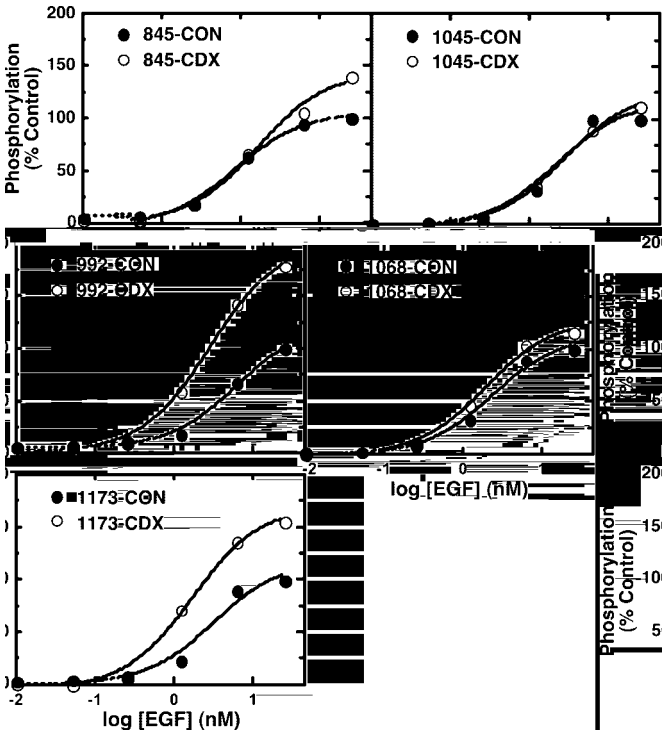


FIG. 4. **Effect of cholesterol on site-specific tyrosine phosphorylation of the EGF receptor.** A431 cells were treated without (control (*CON*)) or with 7.5 mM methyl-β-cyclodextrin (*CDX*) to remove cholesterol and then stimulated with the indicated doses of EGF for 5 min. Cells were lysed and analyzed for receptor phosphorylation by Western blotting with antibodies that recognize phosphorylated tyrosines 845, 992, 1045, 1068, and 1173 on the EGF receptor. Results were quantified by densitometry and subjected to nonlinear curve fitting using GraphPAD Prism. Results from a representative experiment are shown.

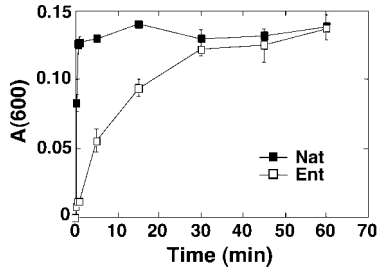


FIG. 5. **Oxidation of *nat*- and *ent*-cholesterol by cholesterol oxidase.** Equal amounts of *nat*-cholesterol (■) and *ent*-cholesterol (□) were subjected to oxidation by cholesterol oxidase as described under "Experimental Procedures." Samples were incubated for the indicated times at 37 °C and analyzed for absorbance at 600 nm.

cholesterol was rapidly oxidized by cholesterol oxidase in a standard cholesterol assay system. The oxidation of *nat*-cholesterol was essentially complete within 30 s under these conditions and showed a $t_{1/2}$ of <10 s. By contrast, *ent*-cholesterol was much more slowly oxidized by cholesterol oxidase under the same conditions. Approximately 30 min were required for complete oxidation of this enantiomer. The reaction had a $t_{1/2}$ of 10 min. Thus, *nat*- and *ent*-cholesterol show clearly distinct behaviors when interacting with a protein that has a specific sterol-binding site.

The capacity of *nat*- and *ent*-cholesterol to interact with other lipids was first examined by comparing the ability of these two enantiomers to condense sphingomyelins in a monolayer system. Because lipid rafts are thought to be held together via cholesterol-sphingolipid interactions, this represents a good measure of the raft-forming capabilities of these enantiomers.

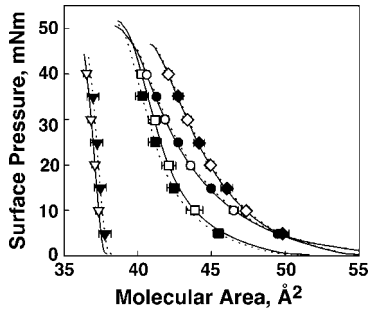


FIG. 6. **Condensation of lipids by *nat*- and *ent*-cholesterol in monolayers.** Surface pressure *versus* average molecular area behavior was measured as described under "Experimental Procedures" for pure *nat*- or *ent*-cholesterol or mixtures of these enantiomers with sphingomyelin. ▽ and ▼, isotherms for pure *nat*- and *ent*-cholesterol, respectively; ○ and ●, isotherms for 30 mol % *nat*- and *ent*-cholesterol, respectively, mixed with 70 mol % egg sphingomyelin; □ and ■, isotherms for 30 mol % *nat*- and *ent*-cholesterol, respectively, mixed with 70 mol % *N*-stearoylsphingomyelin (the naturally occurring form of this lipid); ◇ and ♦, isotherms for 1:1 mixtures of *nat*- and *ent*-cholesterol, respectively, with palmitoyloleoylphosphatidylcholine. Each trace represents the average of three or more experimental isotherms. The standard errors are shown. *mNm*, milli-Newton meter.

Fig. 6 shows the surface pressure-average molecular area isotherms for mixtures of 70 mol % egg sphingomyelin and 30 mol % *nat*- or *ent*-cholesterol (*open* and *closed circles*, respectively). The isotherms were indistinguishable for the two enantiomers. This similarity in behavior was not the result of heterogeneity in the acyl chains present in the egg sphingomyelin mixture. When enantiomerically pure *N*-stearoylsphingomyelin was used in place of the egg sphingomyelin, the isotherms observed for mixtures with *nat*- and *ent*-cholesterol were again indistinguishable (Fig. 6, *open* and *closed squares*). The isotherms of pure *nat*- and *ent*-cholesterol were also identical (Fig. 6, *open* and *closed inverted triangles*). In addition, the force-area behavior of 1:1 mixtures of either *nat*- or *ent*-cholesterol with palmitoyloleoylphosphatidylcholine were the same (Fig. 6, *open* and *closed diamonds*). The condensing effect of cholesterol is a well established indicator of its lateral interaction and nonideal mixing with membrane phospholipids and sphingolipids (29, 34, 35). Therefore, these data indicate that the absolute configuration of the sterol does not affect its interaction with either phosphatidylcholine or sphingomyelin.

*Effects of nat- and ent-Cholesterol on the EGF Receptor Are Similar*—The cholesterol enantiomers were next used in the A431 cell system to determine whether the effect of cholesterol on EGF receptor phosphorylation is the result of general sterol-lipid or stereospecific sterol-protein interactions. A431 cells were depleted of cholesterol and subsequently repleted with either *nat*- or *ent*-cholesterol using complexes of each sterol with methyl-β-cyclodextrin. The data in Table I confirm that treatment of cells with the same concentration of either sterol·cyclodextrin complex added a similar amount of each sterol to cholesterol-depleted cells.

*nat*- and *ent*-cholesterol exhibited similar abilities to reverse the effects of cholesterol depletion on both basal (Fig. 7*A*) and EGF-stimulated (Fig. 7*B*) receptor phosphorylation. Basal EGF receptor phosphorylation was enhanced 6-fold by the depletion of cholesterol. *nat*-Cholesterol reversed this effect, exhibiting an $EC_{50}$ of between 0.07 and 0.2 mM. *ent*-Cholesterol reversed the effects of cholesterol depletion on basal EGF phosphorylation to the same extent as *nat*-cholesterol and exhibited an $EC_{50}$ in the same range as that for the natural enantiomer. *nat*- and *ent*-cholesterol also exhibited similar potencies and efficacies for reversing the enhancement of EGF-stimulated receptor phosphorylation induced by cholesterol depletion (Fig. 7*B*).

To determine whether the similar effects of *nat*- and *ent*-cholesterol were restricted to A431 cells, which express ~3 × $10^6$ EGF receptors/cell, we performed a similar depletion/repletion experiment in Chinese hamster ovary cells, which have been engineered to express a more normal 28,000 EGF receptors/cell. As shown in Fig. 8, cholesterol depletion led to an enhancement of EGF-stimulated receptor phosphorylation. Basal EGF receptor phosphorylation was only slightly affected by cholesterol depletion in this cell line. The effect on EGF-stimulated receptor phosphorylation was reversed to the same ex-

TABLE I

*Cholesterol content of A431 cells treated with methyl-β-cyclodextrin complexes with either enantiomer of cholesterol*

A431 cells were treated without (Control) or with (+Cyclodextrin) 7.5 mM methyl-β-cyclodextrin for 30 min at 37 °C. Cyclodextrin-treated cells were then incubated for 30 min at 37 °C with the indicated concentrations of either *nat*-cholesterol or *ent*-cholesterol in complex with methyl-β-cyclodextrin. The medium was removed, and the cell monolayers were washed twice with cold PBS. Lipids were then extracted, and cholesterol levels were determined using a colorimetric cholesterol assay as described under "Experimental Procedures." Total cellular protein content was determined by the bicinchoninic acid method. The values shown represent the mean ± S.D. of icate determinations.

| Condition | Cholesterol |
|---|---|
| | *μg/mg protein* |
| Control | 32 ± 3 |
| +Cyclodextrin | 20 ± 2 |
| +*nat*-Cholesterol | |
| 0.02 mM | 23 ± 2 |
| 0.07 mM | 26 ± 3 |
| 0.20 mM | 38 ± 4 |
| +*ent*-Cholesterol | |
| 0.02 mM | 25 ± 3 |
| 0.07 mM | 28 ± 3 |
| 0.20 mM | 39 ± 4 |

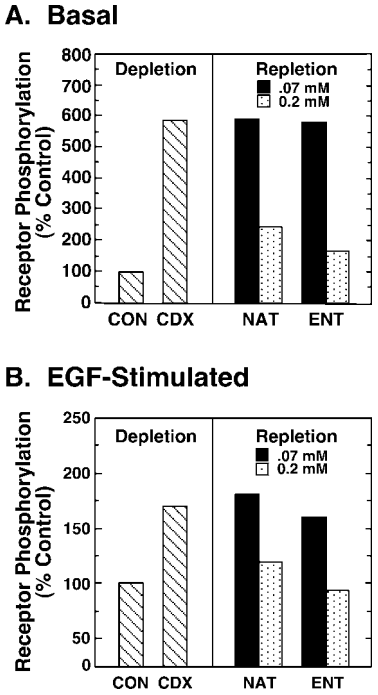


FIG. 7. **Effect of cholesterol repletion on phosphorylation of the EGF receptor.** *A*, A431 cells were treated without (control (*CON*)) or with 7.5 mM methyl-β-cyclodextrin (*CDX*) to remove cholesterol. Some cultures were subsequently repleted with cholesterol by incubation with either *nat*-cholesterol (*NAT*) or *ent*-cholesterol (*ENT*) in complex with methyl-β-cyclodextrin. Concentrations given refer to the amount of cholesterol added. Cells were lysed and analyzed for receptor phosphorylation by Western blotting with anti-phosphotyrosine antibody PY20. Results were quantified by densitometry. *B*, cells were treated as described for *A*, but prior to lysis, cells were stimulated with 5 nM EGF for 5 min.

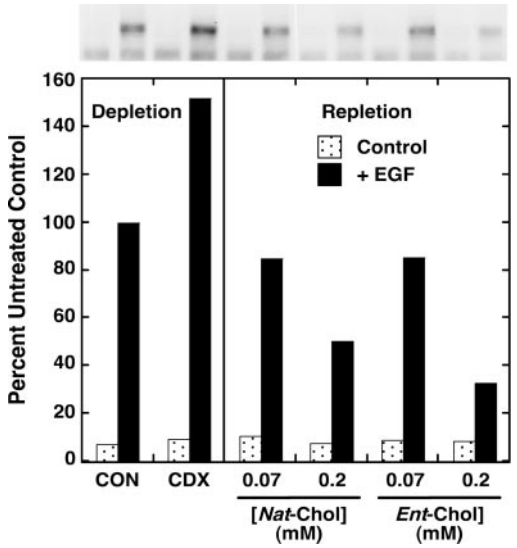


FIG. 8. **Effect of cholesterol repletion on phosphorylation of the EGF receptor.** Chinese hamster ovary cells expressing EGF receptors were treated without (control (*CON*)) or with 7.5 mM methyl-β-cyclodextrin (*CDX*) to remove cholesterol. Some cultures were subsequently repleted with cholesterol by incubation with either *nat*-cholesterol (*Nat-Chol*) or *ent*-cholesterol (*Ent-Chol*) in complex with methyl-β-cyclodextrin. Concentrations given refer to the amount of cholesterol added. Cells were then stimulated with 25 nM EGF for 5 min, lysed, and analyzed for receptor phosphorylation by Western blotting with anti-phosphotyrosine antibody PY20. Results were quantified by densitometry.

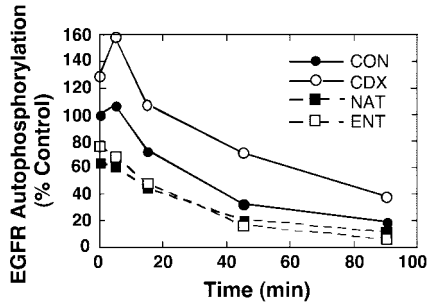


FIG. 9. **Effect of cholesterol depletion and repletion on dephosphorylation of the EGF receptor.** A431 cells were treated without (control (*CON*)) or with 7.5 mM methyl-β-cyclodextrin (*CDX*) to remove cholesterol. Some cultures were subsequently repleted by incubation for 30 min with 0.2 mM *nat*-cholesterol (*NAT*) or *ent*-cholesterol (*ENT*) in complex with methyl-β-cyclodextrin. Cells were stimulated with 1.25 nM EGF for 5 min and washed to remove residual EGF as described under "Experimental Procedures." Cells were incubated in DMEM/BSA for the indicated times and then analyzed for EGF receptor phosphorylation by Western blotting with anti-phosphotyrosine antibody PY20. Results from a representative experiment were quantified by densitometry.

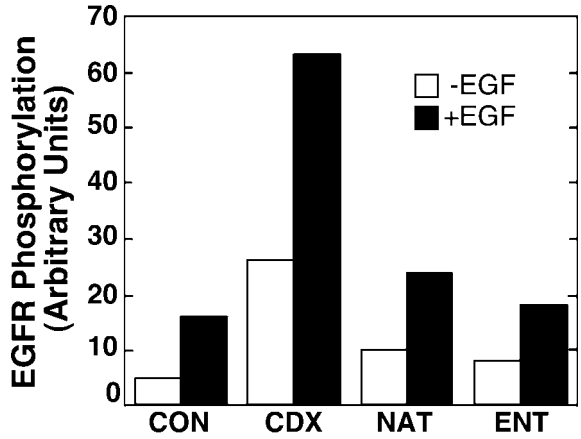


FIG. 10. **Effect of repletion with *nat*- or *ent*-cholesterol on *in vitro* autophosphorylation of the EGF receptor.** A431 cells were treated without (control (*CON*)) or with 10 mM methyl-β-cyclodextrin (*CDX*). Cholesterol-depleted cells were subsequently repleted by incubation for 30 min with 0.2 mM *nat*-cholesterol (*NAT*) or *ent*-cholesterol (*ENT*) in complex with methyl-β-cyclodextrin. Membranes were prepared, and aliquots were subjected to *in vitro* autophosphorylation assays as described under "Experimental Procedures." Results from a representative experiment were quantified by densitometry.

tent and over the same dose range by repletion of the cells with *nat*- and *ent*-cholesterol. Thus, the effects of these enantiomers on EGF receptor autophosphorylation appear to be independent of cell type and the level of receptor expression.

*Cholesterol Affects EGF Receptor Kinase (but Not Phosphatase) Activity*—The observed increase in receptor phosphorylation upon cholesterol depletion could be due either to a decrease in the activity of a protein-tyrosine phosphatase or to an increase in the intrinsic kinase activity of the receptor. To determine whether cholesterol affects the dephosphorylation of the EGF receptor, A431 cells were depleted of cellular cholesterol and then repleted with either *nat*- or *ent*-cholesterol. Following a 5-min stimulation with EGF, cells were washed with a low pH glycine buffer to remove cell-surface EGF. Subsequently, the cells were incubated for increasing lengths of time in EGF-free medium at 37 °C. Cells were lysed and analyzed for EGF receptor phosphorylation.

The data in Fig. 9 show that, as expected, the absolute amount of receptor phosphorylation was higher at all times in cholesterol-depleted cells than in untreated control cells. However, the rate of receptor dephosphorylation was similar in these two treatment groups. Control and cholesterol-depleted cells both exhibited $t_{1/2}$ values for receptor dephosphorylation in the range of 20–25 min. Cells repleted with either *nat*- or *ent*-cholesterol showed similar rates of dephosphorylation, with $t_{1/2}$ values of ~20 min. These data suggest that receptor dephosphorylation is not significantly affected by either the absolute level of cholesterol in the membrane or its absolute configuration.

To determine whether cholesterol levels or the absolute configuration of the sterol affects the intrinsic kinase activity of the EGF receptor, A431 cells were depleted of cholesterol and subsequently repleted with equal amounts of either *nat*- or *ent*-cholesterol. A total membrane fraction was then prepared and assayed for EGF receptor autophosphorylation in an *in vitro* system containing phosphatase inhibitors. The results are shown in Fig. 10. Both basal receptor phosphorylation and EGF-stimulated receptor phosphorylation were increased in membranes prepared from cells depleted of cholesterol. Repletion with *nat*- or *ent*-cholesterol reversed the effect of cholesterol depletion on basal and EGF-stimulated EGF receptor phosphorylation to a similar extent. These data suggest that intrinsic EGF receptor kinase activity is affected by cholesterol levels, but not by the absolute configuration of the sterol.

*nat- and ent-Cholesterol Support Lipid Raft Formation*—Cholesterol depletion disrupts lipid rafts and leads to the loss of the EGF receptor from these low density membrane domains (19, 21). Repletion of cholesterol leads to the reconstitution of lipid rafts and the relocalization of the EGF receptor to these domains (19, 21). An experiment was therefore performed to determine whether *nat*- and *ent*-cholesterol exhibit similar abilities to reconstitute lipid rafts in cholesterol-depleted cells.

A post-nuclear supernatant was prepared from A431 cells that had been depleted of cholesterol and then repleted with *ent*-cholesterol. Assay of this material for cholesterol indicated that 40% of the cholesterol present in these starting membranes was *ent*-cholesterol. Non-detergent lipid rafts were then prepared from the post-nuclear supernatant. In the lipid raft

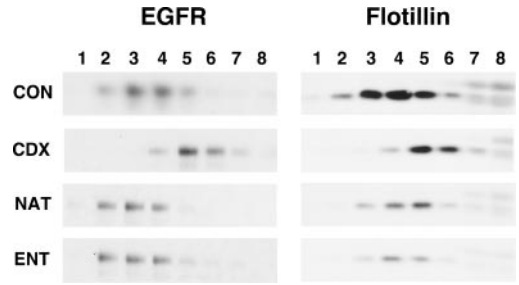


FIG. 11. **Reconstitution of lipid rafts by *nat*- and *ent*-cholesterol.** A431 cells were treated without (control (*CON*)) or with methyl-β-cyclodextrin (*CDX*) for 30 min. Some methyl-β-cyclodextrin-treated cultures were subsequently repleted with cholesterol by incubation for 30 min with 0.2 mM *nat*-cholesterol (*NAT*) or *ent*-cholesterol (*ENT*) in complex with methyl-β-cyclodextrin. Lipid rafts were prepared as described under "Experimental Procedures." Equal aliquots of fractions from the sucrose density gradient were analyzed by SDS-PAGE and Western blotting for the EGF receptor or flotillin.

fraction derived from these starting membranes, 41% of the cholesterol was determined to be *ent*-cholesterol. In a separate experiment, a 1% Triton X-100 extract was made from cells that had been depleted of cholesterol and repleted with *ent*-cholesterol. In this experiment, 54% of the cholesterol in the Triton X-100 extract was *ent*-cholesterol. After flotation in a sucrose gradient, the resulting Triton X-100-resistant lipid rafts were assayed and found to contain 60% *ent*-cholesterol. These data indicate that *ent*-cholesterol partitions into both non-detergent and Triton X-100-resistant lipid rafts in direct proportion to its concentration in the starting membranes.

A431 cells were depleted of cholesterol using methyl-β-cyclodextrin and repleted with equal amounts of either *nat*- or *ent*-cholesterol. Non-detergent lipid rafts were then prepared by sucrose density gradient centrifugation, and the distribution across the gradient of the EGF receptor and flotillin, a lipid raft marker protein, was assessed. The results are shown in Fig. 11. As expected, both the EGF receptor and flotillin selectively partitioned into the low density portion of the gradient in untreated cells. Depletion of cholesterol using methyl-β-cyclodextrin resulted in the loss of both proteins from the low density region of the gradient and their recovery in higher density fractions. Repletion of cells with *nat*-cholesterol reconstituted the lipid rafts, restoring the partitioning of flotillin and the EGF receptor into the low density fractions of the gradient. Similarly, repletion with *ent*-cholesterol led to the movement of both flotillin and the EGF receptor back into the low density region of the gradient. These data suggest that *nat*- and *ent*-cholesterol have similar capacities to reconstitute lipid rafts and to promote partitioning of the EGF receptor into these low density membrane domains.

## DISCUSSION

*Cholesterol Depletion*—The ability of cholesterol depletion to enhance EGF receptor phosphorylation has been observed by several groups (17–19). In this study, we provide additional details regarding the characteristics of cholesterol-modulated EGF receptor phosphorylation and provide insight into the molecular basis of this phenomenon.

In A431 cells, cholesterol depletion by methyl-β-cyclodextrin was found to enhance both basal and EGF-stimulated receptor tyrosine phosphorylation. The increase in ligand-dependent phosphorylation of the EGF receptor observed in these studies was found to be due to an increase in the maximal level of receptor phosphorylation rather than a change in the $EC_{50}$ for EGF. Only an ~10% increase in EGF receptor number was observed in A431 cells following cholesterol depletion. Because this is substantially smaller than the percent increase in EGF-stimulated autophosphorylation observed in our experiments, the enhancement of EGF receptor phosphorylation cannot be attributed solely to a rise in surface-exposed EGF receptors.

We (19) and others (18, 20) have observed that cholesterol depletion leads to an increase in the number of cell-surface EGF receptors in a variety of other cell types. Evidence suggests that this increase is due to the unmasking of cryptic receptors present on the cell surface (18, 19). Recent structural studies of the extracellular domain of the EGF receptor offer insight into a possible molecular mechanism for this effect. The unliganded EGF receptor exists in a bent autoinhibited configuration that is stabilized by interactions between loops in the second and fourth extracellular subdomains (36). It apparently binds EGF only weakly (36). This bent inactive conformation is likely in equilibrium with an open configuration that binds EGF with high affinity and is capable of forming functionally active receptor dimers (37, 38). EGF shifts this equilibrium from the inactive to the active configuration of the receptor by binding to and stabilizing the open form (36, 38). The observation that cholesterol depletion results in an increase in EGF binding with no change in the amount of receptor protein detectable at the cell surface (19) suggests that high cholesterol levels may stabilize the intramolecular interactions, increasing the fraction of the EGF receptor that is in the inactive conformation. Cholesterol depletion would reduce this intramolecular stabilization, allowing the receptor to more readily adopt the open conformation necessary for tight EGF binding.

The increase in EGF receptor phosphorylation in response to cholesterol removal could be due to an increase in the intrinsic kinase activity of the receptor or to a decrease in the rate of receptor dephosphorylation. Cell culture experiments demonstrated that the $t_{1/2}$ for receptor dephosphorylation was similar in control and cyclodextrin-treated cells. By contrast, following cholesterol depletion, EGF receptor autophosphorylation was enhanced in *in vitro* experiments, in which kinase activity was assayed in the presence of phosphatase inhibitors. Together, these data suggest that it is primarily an increase in intrinsic receptor kinase activity that gives rise to enhanced receptor phosphorylation in intact cells depleted of cholesterol. This is consistent with recent observations made in NIH 3T3 cells (19).

A novel finding with respect to the increase in EGF receptor phosphorylation following cholesterol depletion is that there was a selective increase in the phosphorylation of some sites, with little or no effect on the phosphorylation of other sites. Tyrosines 992, 1045, 1068, and 1173 are all known sites of EGF receptor autophosphorylation (39, 40). However, the phosphorylation of tyrosines 992 and 1173 was routinely enhanced 1.5–2-fold by cholesterol depletion, whereas the phosphorylation of tyrosines 1045 and 1068 was minimally affected by cholesterol withdrawal. The observation that there was not a uniform increase in the phosphorylation of all sites available to the EGF receptor kinase implies either that the specificity of the kinase was altered or that the accessibility of the kinase to the individual sites differed in control and cholesterol-depleted cells. Cholesterol depletion is accompanied by the disruption of lipid rafts and the loss of the EGF receptor from this compartment (19, 21). It is therefore tempting to speculate that the intracellular domain of the EGF receptor adopts a different conformation when it is outside of lipid rafts than when it is localized to this compartment. This would give rise to differences in the accessibility of the various sites of autophosphorylation and would be reflected in the overall pattern of receptor phosphorylation.

Tyrosine 845 is a site on the EGF receptor known to be phosphorylated by $pp60^{src}$ (41, 42). The observation that the phosphorylation of this site was selectively enhanced by cho-

lesterol depletion implies that either this site becomes more accessible to pp60$^{src}$ in cholesterol-depleted cells or that cholesterol depletion enhances the activation of pp60$^{src}$ in response to EGF. Phosphorylation of Tyr$^{845}$, which is in the activation loop of the kinase domain, is associated with an increase in the kinase activity of the EGF receptor (41). It is therefore possible that the increase in the intrinsic kinase activity of the EGF receptor that occurs following cholesterol depletion is a result of the enhanced phosphorylation of the receptor at Tyr$^{845}$. Additional studies will be required to clarify the molecular basis of this effect.

Specific phosphorylated tyrosine residues on the EGF receptor serve as binding sites for adaptor proteins and enzymes (43–46). The finding that a decrease in membrane cholesterol levels leads to selective increases in the phosphorylation of specific tyrosine residues suggests that cholesterol depletion could preferentially enhance signaling via specific pathways. For example, Shc binds selectively to Tyr$^{1173}$, a residue whose phosphorylation is selectively enhanced in cholesterol-depleted cells (44). Thus, the MAPK pathway would be expected to be activated to a greater extent in cyclodextrin-treated cells than other pathways that depend on tyrosine residues whose phosphorylation is not selectively increased. Interestingly, the phosphorylation of Tyr$^{1045}$, the binding site for the ubiquitin ligase c-Cbl (46), which down-regulates the EGF receptor, was not enhanced in cyclodextrin-treated cells. This suggests that the increased kinase activity of the receptor is not counterbalanced by a Cbl-mediated increase in receptor internalization and down-regulation. The up-regulation of kinase activity without a concomitant increase in the negative regulatory mechanisms could serve to further enhance the relative signaling capacity of the EGF receptor in cholesterol-depleted cells.

*Enantiomers of Cholesterol*—The ability of cholesterol to modulate EGF receptor function could arise from several sources, including alterations in specific sterol-protein interactions, or from more general effects of the sterol on membrane structure. To distinguish between these two possibilities, the enantiomer, or mirror image, of cholesterol was synthesized and used in experiments in which *nat*-cholesterol was replaced by its enantiomer, *ent*-cholesterol. Preliminary experiments using cholesterol oxidase demonstrated that an enzyme possessing a specific binding site for cholesterol is capable of distinguishing between the enantiomers of cholesterol. Such enantioselective interactions appear to be absolutely required for viability of organisms, as our previous work has shown that substitution of *ent*-cholesterol for *nat*-cholesterol in the growth medium of *Caenorhabditis elegans* results in 100% lethality in second generation animals (47). Thus, proteins and intact organisms can distinguish *nat*-cholesterol from *ent*-cholesterol.

By contrast, in the monolayer experiments in which sterol-lipid interactions were examined, the enantiomers of cholesterol were indistinguishable in terms of their ability to condense sphingomyelin and phosphatidylcholine. This result is consistent with recent studies that have found that artificial bilayers containing sphingomyelin and either *nat*- or *ent*-cholesterol are indistinguishable as assessed by differential scanning calorimetry, x-ray diffraction, and neutral buoyant density measurements (48). In addition, we and others (49–52) have found no enantioselectivity in the interaction of cholesterol with phospholipids. Thus, with respect to their interactions with membrane lipids, *nat*- and *ent*-cholesterol appear to function similarly.

We took advantage of the difference in the behavior of the enantiomers of cholesterol in situations involving specific molecular recognition or more general sterol-lipid interactions to gain insight into the molecular mechanism underlying the effects of cholesterol on EGF receptor function. By replacing *nat*-cholesterol with *ent*-cholesterol in cholesterol-depleted cells, it was possible to compare the effect of the two enantiomers on EGF receptor phosphorylation.

*ent*-Cholesterol was found to partition into lipid rafts in direct proportion to its concentration in total cell membranes, indicating that it behaves similarly to *nat*-cholesterol in its ability to distribute into cell membranes. Furthermore, our studies indicate that *nat*- and *ent*-cholesterol have equal abilities to reverse the increase in EGF receptor phosphorylation observed following cholesterol depletion. Both the extent of the reversal and the dose of the sterol required to observe reversal were the same for *nat*- and *ent*-cholesterol. In addition, in cholesterol-depleted cells, the EGF receptor and the raft marker protein flotillin shifted out of the low density portion of sucrose density gradients, reflecting the disruption of cholesterol-enriched lipid rafts (19, 21). *nat*- and *ent*-cholesterol were equally capable of reconstituting the lipid rafts, as evidenced by the movement of the EGF receptor and flotillin back into the low density portion of the gradient following repletion with either sterol. Thus, no distinction was made at any level between *nat*- and *ent*-cholesterol. These observations indicate that the effect of cholesterol on EGF receptor function and raft integrity is not enantioselective.

Because proteins that possess specific binding sites for cholesterol appear to readily distinguish between *nat*- and *ent*-cholesterol, whereas sterol-lipid interactions appear to be non-enantioselective, the present observations suggest that the effects of cholesterol on EGF receptor function are due to effects of the sterol on the physical properties of the membrane. We have shown previously that sterol analogs such as 25-hydroxycholesterol and 7-ketocholesterol differentially affect EGF receptor function when exchanged into cell membranes (21). Because of their additional chemical groups, these analogs impart different properties on the membrane than cholesterol. However, these analogs have an overall molecular shape that is more similar to that of *nat*-cholesterol than *ent*-cholesterol. Nonetheless, the receptor discriminated among these sterol analogs that have similar shapes, but impart different properties on the membrane. The present findings show that the receptor does not discriminate between *nat*- and *ent*-cholesterol, which have different shapes, but impart similar properties on the membrane. Thus, there appears to be a correlation between the ability of a sterol to alter membrane properties (as compared with cholesterol) and its ability to alter EGF receptor function (as compared with cholesterol). Therefore, although we cannot rule out the possibility that cholesterol binds in a non-enantioselective manner to the EGF receptor, we favor the interpretation that the effects of cholesterol on EGF receptor function are due to cholesterol-induced changes in the physical properties of the cell membrane. It seems likely that any non-enantioselective binding of cholesterol to the EGF receptor would occur at hydrophobic sites that are not specifically regulatory in nature. Such nonspecific interactions would probably be dependent on the lipid composition and physical properties of the membrane and hence would be encompassed in the general membrane level effects of sterols on EGF receptor function.

Because cholesterol is critical for inducing the formation of lipid rafts and because EGF receptors are normally localized to lipid rafts, it is possible that the effects of cholesterol on receptor function are ultimately due to the ability of cholesterol to induce lateral domain formation in membranes. The high concentration of cholesterol within lipid raft domains may affect EGF receptor conformation and function through lipid-based mechanisms involving changes in membrane fluidity,

thickness, or stabilization of a particular conformation of the protein.

In summary, our data indicate that cholesterol depletion induces an increase in the intrinsic kinase activity of the EGF receptor and leads to enhanced site-specific phosphorylation of the receptor. The observation that changes in membrane cholesterol levels alter the pattern of EGF receptor phosphorylation suggests that the extent of activation of individual downstream signaling pathways by EGF could differ depending on the level of cholesterol in the cell membrane. As a variety of pathological conditions, including atherosclerosis and type C1 Niemann-Pick disease (53), lead to alterations in cellular cholesterol levels, it is possible that changes in hormonal signaling occur in diseased cells as a result of underlying problems in cholesterol trafficking. Our data also suggest that the effects of cholesterol on receptor signaling are most likely due to the ability of cholesterol to interact with membrane lipids and possibly promote the formation of lateral membrane domains. Through this mechanism, increased membrane cholesterol content could have widespread effects on membrane protein function.

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