Sequestration of Epidermal Growth Factor Receptors in Non-caveolar Lipid Rafts Inhibits Ligand Binding*

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Cholesterol depletion has been shown to increase mitogen-activated protein kinase activation in response to stimulation with epidermal growth factor (EGF) (Furuchi, T., and Anderson, R. G. W. (1998) J. Biol. Chem. 273, 21099–21104). However, the underlying mechanisms are unknown. We show that cholesterol depletion increases EGF binding, whereas cholesterol loading lowers EGF binding. Based on binding analyses, we demonstrate that the observed changes in EGF binding are caused by alterations in the number of EGF receptors available for ligand binding, whereas the affinity of the receptor for EGF remains unaltered. We also show by immunofluorescence that in unstimulated cells the EGF receptor is localized in non-caveolar lipid rafts containing the ganglioside GM1 and that patching of these rafts by cholera toxin B-chain causes co-patching of EGF receptors. Experiments with solubilization in different detergents at 4 °C show that the association of the EGF receptor with these rafts is sensitive to Triton X-100 extraction but insensitive to extraction with another non-ionic detergent, Brij 58. Furthermore, experiments with cholesterol-depleted cells show that the association is cholesterol-dependent. We propose that non-caveolar lipid rafts function as negative regulators of EGF receptor signaling by sequestering a fraction of the EGF receptors in a state inaccessible for ligand binding.

Cholesterol- and glycosphingolipid-enriched membrane domains function as signaling platforms providing the structural environment for interactions between ligands, plasma membrane receptors, and downstream molecules. This is particularly well established for non-caveolar domains, the so-called rafts, but many reports also point to a similar function of caveolae (see Refs. 2 and 3 for reviews).

Several studies have reported that in unstimulated cells, EGF receptors are concentrated in plasma membrane caveolae and that they move out of caveolae following stimulation with EGF (1, 4). However, these conclusions are based on studies where caveolae were isolated by subcellular fractionation via density centrifugations. Because the buoyancy of caveolae and non-caveolar lipid rafts is believed to be the same, such fractionation methods may not yield a pure caveola fraction but rather a fraction containing both caveolae and non-caveolar lipid rafts. Indeed, a study based on immunoisolation of caveolae from such membrane fractions has reported that the unstimulated EGF receptor is localized in low buoyancy non-caveolar membrane domains (5). Although the exact localization of EGF receptors in the plasma membrane is uncertain, it is clear that cholesterol affects the function of the receptor. It has been shown that cholesterol depletion causes EGF-mediated hyperactivation of mitogen-activated protein kinases and increased DNA synthesis (1).

Disruption of caveolae and lipid rafts by cholesterol depletion has been shown to influence the signaling from many plasma membrane receptors other than the EGF receptor, including receptor protein-tyrosine kinases like the insulin receptor, the T-cell antigen receptor, and several G-protein-coupled receptors (1, 6–11). However, the exact mechanisms whereby cholesterol depletion exerts its effect on receptor signaling are in most cases unknown. In case of the EGF receptor, it has been reported that direct interactions between the EGF receptor and the caveolin scaffolding domain inhibit autophosphorylation of the EGF receptor (12). It has therefore been suggested that caveolin serves as a negative regulator of EGF receptor mitogenic signaling and that disruption of caveolae by cholesterol depletion leads to loss of caveolin-mediated inhibition of EGF receptor signaling (13). However, in light of the remaining uncertainty concerning whether EGF receptors are localized in caveolae, it is unclear whether the effect of cholesterol depletion on EGF receptor signaling can be attributed to disruption of a caveolin-EGF receptor interaction.

It is known that in some cases cholesterol can modulate ligand binding to membrane receptors, thereby affecting signal transduction. Studies of some G-protein-coupled receptors, oxytocin receptor, cholecystokinin receptor, and the galanin receptor GalR2 have shown that cholesterol depletion with methyl-β-cyclohextrin (mβCD) markedly decreases ligand binding to these receptors (8, 9).

We show that cholesterol depletion increases ligand binding to the EGF receptor, whereas cholesterol loading decreases ligand binding. The modulation of EGF receptor ligand binding is because of changes in the number of binding sites, Bmax, whereas the affinity of the receptor for EGF, Kd, remains constant. By confocal microscopy we show that the EGF receptor is localized in membrane domains containing the ganglioside GM1. A small fraction of these EGF receptor-containing membrane domains may be caveolae, but the majority are non-caveolar membrane domains. Furthermore, we show that the EGF receptor is associated with Triton X-100-soluble, Brij 58-insoluble membrane domains and that this association is cholesterol-dependent. We therefore propose that non-caveolar rafts are negative regulators of EGF receptor ligand binding.
**EXPERIMENTAL PROCEDURES**

All reagents were purchased from Sigma unless otherwise indicated.

**Cell Culture**—HeLa, A431, and HEp2 cells were grown in T25 or T75 flasks (Nalge Nunc International Corp., Naperville, IL) at 37 °C, 5% CO₂ in DMEM supplemented with 10% fetal calf serum, 2 mM glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin (all reagents from Invitrogen). For 125I-EGF binding experiments, the cells were grown in 12-well Nunclon Multidishes (Nalge Nunc International Corp., Naperville, IL) for 24 h (HeLa and HEp2 cells) or for 48 h (A431 cells) prior to experiments. For immunofluorescence, HeLa cells were grown on 4-chamber Labtek chamber slides (Nalge Nunc International Corp., Naperville, IL) for 24 h before the experiments.

**Cholesterol Depletion, Cholesterol Loading, Filipin Treatment, and Energy Depletion**—All of the experiments with mCD, mCD-cholesterol, mCD filamentin, or mCD-filipin were done using DMEM/HEPES medium containing 0.2% BSA. For cholesterol depletion, cells were incubated with medium containing the indicated concentration of mCD for 30 min at 37 °C. For cholesterol loading, cells were incubated with medium containing the indicated concentration of an mCD-cholesterol complex for 30 min at 37 °C. After the incubations, cells were rinsed 3× with warm medium before further experiments were performed. For treatment with filipin, filipin was dissolved to 20 μg/ml in DMSO, and this stock solution was frozen at −20 °C. For experiments, cells were incubated with this stock diluted in medium to give the concentration of filipin indicated.

For energy depletion, cells were incubated with depletion medium (DMEM/HEPES, 50 mM 2-deoxyglucose, 10 mM NaF) for 30 min at 37 °C. Further incubations with mCD were performed using depletion medium. For energy depletion experiments, cells were energy depleted or left as control and thereafter incubated with tetramethylrhodamine-conjugated EGF or transferrin (both from Molecular Probes, Leiden, the Netherlands) in depletion medium or control medium for 30 min at 37 °C. The cells were analyzed by confocal microscopy to confirm that endocytosis was blocked in the energy-depleted cells compared with control cells.

**Protein Determination**—Protein concentrations were determined using the Bio-Rad DC protein assay as described by the manufacturer (Bio-Rad).

**Cholesterol Determination**—Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% SDS, 2 mM EDTA, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin), and the lysates were homogenized using a 21-gauge needle on a 1-ml syringe. Cholesterol was determined spectrophotometrically as reported previously (11) using the Infinity Cholesterol Assay kit from Sigma.

**125I-EGF Binding Analysis**—For binding analyses, cells were grown in 12-well Nunclon Multidishes, treated as described or left as control cells, and incubated with 1 mM 125I-EGF (Amersham Biosciences AB) in DMEM/HEPES with 0.2% BSA for 1 h on ice. The incubation medium was removed, and the cells were washed 3× with ice-cold phosphate buffer (800 μl of 1 mM NaOH). The hydrolized cells were transferred to tubes for γ-counting.

For determination of Kᵄₐ and Bₘₐₓ values, cells were grown in 12-well Nunclon multidishees, treated as described or left as control cells, and incubated with increasing concentrations of 125I-EGF for 4 h on ice in the absence (total binding) or presence (nonspecific binding) of 200 nM unlabeled EGF (Calbiochem). The incubation medium was removed, and the cells were washed 3× with ice-cold PBS and hydrolyzed in 200 μl of 1 M NaOH. The hydrolized cells were transferred to tubes for γ-counting. All binding data were correlated to total amount of cellular protein. The specific binding was calculated as total binding minus nonspecific binding, and the data were analyzed by nonlinear curve fitting to the equation, bound = Bₘₐₓ × concentration/Kᵄₐ + concentration (StatSoft, Tulsa, OK). Nonspecific binding was less than 5% of total binding.

**Detergent Lysis and Centrifugation**—Cells were cholesterol-depleted with the indicated concentration of mCD or left as control cells, rinsed 3× with PBS, and harvested in ice-cold PBS by scraping with a rubber policeman. The cells were pelleted by centrifugation, resuspended in PBS, and split into 1-3 aliquots. The cells in each aliquot were pelleted by centrifugation, resuspended in ice-cold PBS (150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl, pH 7.4, 100 μg/ml phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin) containing 0.5% detergent (Triton X-100, Brij 56, or Brij 58) by gentle mixing at 4 °C for 30 min. The lysates were centrifuged at 800 × g for 10 min at 4 °C, and the postnuclear supernatant was transferred to a new tube. The lysates were centrifuged at 16,000 × g for 20 min at 4 °C, and the supernatant was transferred to a new tube, and the pellet containing insoluble membrane domains was resuspended in buffer A containing 0.5% of the appropriate detergent.

**Western Blotting**—Pellets and supernatants of the centrifuged cell lysates were separated on 4–12% Bis-Tris acrylamide gels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences AB). The membranes were blocked in blocking buffer (5% BSA, 5% Bio-Rad) and incubated with primary antibodies and secondary antibodies were bound to the membranes in blocking buffer. The antibodies used were rabbit polyclonal anti-caveolin-1 (Transduction Laboratories and BD Pharmingen) diluted 1:3000, mouse monoclonal anti-EGF receptor diluted 1:1000, horseradish peroxidase swine anti-rabbit diluted 1:3000, and horseradish peroxidase goat anti-mouse (Amersham Biosciences AB). Membranes were washed 4 times in PBS with 0.1% Tween 20 between antibody incubations. Immunosignals were visualized using enhanced chemiluminescence reagent exposing Hyperfilm (Amersham Biosciences AB).

**Immunofluorescence Microscopy**—For semi-quantitative labeling of surface EGF receptors, cells were grown on chamber slides were cholesterol-depleted or cholesterol-loaded as described and fixed with 2% paraformaldehyde in PBS for 15 min at room temperature. The fixed cells were blocked in blocking buffer (5% goat serum (Dako, Glostrup, Denmark) and 1% BSA, in PBS) and the unpermeabilized cells incubated with primary anti-EGF receptor antibodies and secondary fluorescent antibodies to label surface EGF receptors. The average cellular fluorescence intensity in control cells or treated cells, corresponding to the amount of surface EGF receptors, was determined in the confocal microscope by analyzing 8–10 groups of 5–15 cells using a ×20 objective and a fully open pinhole. The average cellular fluorescence was calculated by dividing the total fluorescence minus background fluorescence with the number of cells analyzed in each group and finally averaging the obtained cellular fluorescence values for the 8–10 different groups.

For co-localization of the EGF receptor with caveolin-1 and GM1 cells grown on chamber slides were first incubated with a cholesterol B-chain (CT-B) Alexa 594 conjugate (10 μg/ml) (Molecular Probes, Leiden, the Netherlands) dissolved in DMEM/HEPES, 0.2% BSA, for 1 h on ice to label GM1. Cells were then washed 3× in cold PBS and fixed with 2% paraformaldehyde in PBS for 10 min on ice and thereafter 10 min at room temperature. Cells were blocked and permeabilized in blocking buffer containing 0.5% saponin, incubated with 20 μg/ml secondary antibodies were bound to the membranes in blocking buffer containing 0.2% saponin for 1 h, washed 3 times with PBS, and incubated with secondary fluorescent antibodies for 30 min.

Patchig of GM1 was performed as follows: cells grown on chamber slides were incubated with CTB-Alexa 594 for 1 h at 12 °C, washed, incubated with an anti-cholera toxin polyclonal antibody for 1 h at 12 °C, washed, and fixed in 2% paraformaldehyde. The fixed cells were then labeled using confocal microscopy by analyzing 8–10 groups of 5–15 cells using a ×20 objective and a fully open pinhole. The average cellular fluorescence was calculated by dividing the total fluorescence minus background fluorescence with the number of cells analyzed in each group and finally averaging the obtained cellular fluorescence values for the 8–10 different groups.

**RESULTS**

**Cholesterol Depletion Increases EGF Binding**—To investigate the effect of cholesterol depletion on EGF receptor ligand binding, HeLa cells were cholesterol-depleted using mCD. As can be seen from Fig. 1A, a 30-min extraction with increasing concentrations of mCD removed increasing amounts of cellular cholesterol, as measured by a spectrophotometric cholesterol assay. The influence of cholesterol on EGF binding was analyzed by incubation for 30 min in ice-cold PBS with 1 mM 125I-EGF. Cholesterol depletion of HeLa cells with increasing concentrations of mCD led to an increase in EGF binding (Fig. 1B). The same effect was seen on A431 and HEp2 cells (unpublished observations).

Cholesterol depletion with mCD is known to disrupt caveolae (15, 16). Because several studies (1, 4) have reported that the EGF receptor is located in caveolae, the effect of cholesterol depletion on EGF binding could be due to disruption of caveolae. Another way to disrupt caveolae is by incubating the cells...
with the cholesterol-binding drug filipin, which does not remove cholesterol from the membrane but causes it to aggregate via cross-linking (17). We therefore incubated HeLa cells with 2 or 5 \( \mu \text{g/ml} \) filipin for 30 min followed by binding of \(^{125}\text{I}-\text{EGF} \) on ice. No changes in EGF binding were seen following filipin treatment (Fig. 1C). It thus appears that the disruption of caveolae is not sufficient to cause increased EGF binding but that the increase in EGF binding following cholesterol extraction with \( \text{mBCD} \) is due to the removal of cholesterol from the plasma membrane.

\( K_d \) for EGF Binding Remains Unchanged following Cholesterol Depletion, whereas \( B_{\text{max}} \) Is Increased—We next investigated whether the effect of cholesterol depletion on EGF binding was due to changes in the affinity of the receptor for the ligand (\( K_d \)) or to changes in the total number of binding sites (\( B_{\text{max}} \)). Cells were left untreated or incubated with 5 \( \mu \text{M mBCD} \), and subsequently increasing concentrations of \(^{125}\text{I}-\text{EGF} \) were bound to the cells on ice for 4 h. Fig. 2A shows the binding curves obtained (compare middle and upper curves). The best fit values for \( K_d \) and \( B_{\text{max}} \) were determined by nonlinear regression. Although the value for \( K_d \) remained unchanged, \( B_{\text{max}} \) was increased by cholesterol depletion compared with control cells (Fig. 2B). This means that cholesterol depletion increases the number of EGF-binding sites on the plasma membrane.

Because cholesterol depletion has been shown to inhibit clathrin-dependent endocytosis (16), one possible explanation for the increase in \( B_{\text{max}} \) following cholesterol depletion could be an inhibition of a basal rate of endocytosis of EGF receptors. Alternatively, cholesterol depletion could induce exocytosis, thereby recruiting receptors from an intracellular pool. Because both endo- and exocytosis are energy-requiring processes, we therefore investigated the energy requirement of the cholesterol depletion-induced increase in EGF binding. HeLa cells were energy-depleted for 30 min; thereafter cholesterol was depleted with 5 \( \mu \text{M mBCD} \) for 30 min in energy depletion medium, and subsequently \(^{125}\text{I}-\text{EGF} \) was bound on ice. The energy depletion of the cells was confirmed by inhibition of internalization of fluorescent transferrin or fluorescent EGF (not shown). In energy-depleted cells, the effect of cholesterol depletion on ligand binding reflects the effect on receptors already present in the plasma membrane, because both endo- and exocytosis are abolished.

As can be seen from Fig. 2C, energy depletion alone did not alter the binding of \(^{125}\text{I}-\text{EGF} \). Following cholesterol depletion of energy-depleted cells, an increase in EGF binding was seen similar to the increase in cholesterol-depleted control cells. This rules out changes in endo- and exocytosis as mechanisms for the cholesterol-regulated changes in EGF-binding sites on the plasma membrane. It thus appears that cholesterol depletion increases the accessibility of EGF receptors in the plasma membrane for ligand binding.

Cholesterol Loading Decreases \( B_{\text{max}} \) for EGF Binding, whereas \( K_d \) Remains Unchanged—Because the above results indicate that cholesterol depletion increases the accessibility of EGF receptors for EGF binding, we hypothesized that cholesterol loading might have the opposite effect, i.e., to decrease the accessibility of EGF receptors for ligand binding. To test this, HeLa cells were loaded with cholesterol by incubation with increasing concentrations of an \( \text{mBCD}-\text{cholesterol} \) complex for 30 min. Fig. 3A shows that incubation with increasing amounts of \( \text{mBCD}-\text{cholesterol} \) led to an increase in cellular cholesterol. Incubation of cells with \(^{125}\text{I}-\text{EGF} \) on ice showed that this increase in cellular cholesterol inhibits EGF binding (Fig. 3B).

To determine whether this decrease in EGF binding was also due to a change in \( B_{\text{max}} \), as is the case for the binding changes following cholesterol depletion, we next performed a ligand binding analysis of cholesterol-loaded cells. Cells were cholesterol-loaded by incubation for 30 min with \( \text{mBCD}-\text{cholesterol} \), and subsequently \( K_d \) and \( B_{\text{max}} \) values for \(^{125}\text{I}-\text{EGF} \) were determined as described for cholesterol-depleted cells. The binding curves for control and cholesterol-loaded cells are shown in Fig. 2A (compare lower and middle curves). As can be seen from Fig. 2B, cholesterol loading decreases \( B_{\text{max}} \) for EGF binding, whereas \( K_d \) remains unchanged.

The increase in \( B_{\text{max}} \) following cholesterol loading could be due to induction of endocytosis of EGF receptors, thereby lowering the number of receptors on the cell surface. To further control that the observed changes in \( B_{\text{max}} \) values following cholesterol loading or cholesterol depletion were not due to an altered number of EGF receptors on the cell surface, we next measured the amount of surface EGF receptors on control, cholesterol-depleted, and cholesterol-loaded cells. The cells were grown on chamber slides, cholesterol-depleted, cholesterol-loaded, or left as control cells, and fixed. The fixed cells were left unpermeabilized and immunostained with anti-EGF receptor antibodies to label only surface EGF receptors. The average cellular fluorescence, corresponding to the amount of EGF receptors on the cell surface, was then measured by quantitative fluorescence microscopy as described under “Experimental Pro-
as can be seen from Fig. 3C, no change in the amount of surface EGF receptors was seen following cholesterol deple-
tion or loading.

Plasma Membrane EGF Receptors Co-localize with the Ga-
glioside GM1—Because cholesterol modulates EGF receptor
ligand binding, we next investigated the localization of EGF
receptors in the plasma membrane. As mentioned above, the
EGF receptor has been reported to localize to caveolae. We
therefore looked at the localization of the EGF receptor rel-
ative to caveolae by immunofluorescence. Cells were fixed and
stained with anti-EGF receptor and anti-caveolin-1 and exam-
ined by confocal microscopy. Whereas caveolin-1 showed the
characteristic dotted distribution typical of caveolae, EGF re-
ceptors were much more evenly distributed throughout the
plasma membrane (see Fig. 4, A–C). Although it cannot be
excluded that some EGF receptors were present in caveolae, by
far the majority of EGF receptors was present outside caveolae,
suggesting that the receptor could be associated with non-
caveolar rafts.

To visualize the location of EGF receptor not present in
caveolae, we investigated the localization of EGF receptor rel-
relative to different markers of lipid rafts by immunofluorescence.

Between EGF receptor and Reggie-1/Flotillin-2 or between EGF
receptor and the glycosylphosphatidylinositol-anchored protein
placental alkaline phosphatase (not shown). Reggie-1/Flotil-
lin-2 is a constituent of non-caveolar micropatches in neuronal
and non-neuronal cells (18). Placental alkaline phosphatase is
an often used marker of raft domains (19). However, the cells
we analyzed expressed very little of both Reggie-1/Flotillin-2
and placental alkaline phosphatase, and these raft markers
may therefore not be suitable. We therefore investigated the
localization of the EGF receptor relative to the ganglioside
GM1. At the electron microscopic level, GM1 has been detected
both in caveolae and in patches distinct from caveolae, presum-
ably raft structures, with a diameter of 0.1 μm (20), and exper-
iments with antibody-induced cross-linking have shown that
GM1 co-patches with raft markers such as glycosylphosphati-
dylinositol-anchored proteins (19). As a marker of GM1 we used
fluorescent cholera toxin B-chain (CT-B). Fluorescent CT-B
was bound to HeLa cells on ice; the cells were fixed and sub-
sequently labeled for EGF receptor and caveolin-1. We found
that some GM1 co-localized with caveolin-1 in caveolae,
whereas the majority of GM1 was located outside caveolae (not
shown). Interestingly, a very strong co-localization was seen
between plasma membrane EGF receptors and GM1 (Fig. 4,

Fig. 2. Cholesterol depletion or loading changes B_max by a non-energy requiring process. A, HeLa cells were cholesterol-depleted with
5 mM mβCD or cholesterol-loaded with 1 mM mβCD-cholesterol for 30 min at 37 °C or left as control. Increasing concentrations of [125I]-EGF were
bound for 4 h on ice, and the cells were washed and lysed in 1 M NaOH. All samples were counted in a γ-counter. The curves show pmol of [125I]-EGF
bound/mg total cellular protein as a function of [125I]-EGF for a representative experiment. Data were analyzed by nonlinear regression to obtain
values for B_max and K_d. B, table shows the calculated values for B_max and K_d given as means ± S.D. from three independent experiments. C, HeLa
cells were energy-depleted by incubation with 50 mM 2-deoxyglucose, 10 mM NaN_3 for 30 min at 37 °C and subsequently cholesterol-depleted with
5 mM mβCD for 30 min at 37 °C as indicated. The cells were thereafter incubated with 1 nM [125I]-EGF on ice for 1 h, washed, and lysed in 1 M NaOH.
All samples were counted in a γ-counter. Values are given as percentage of control. Means ± S.D. are from three independent experiments. **, different from control, p < 0.01; *, different from control, p < 0.05.
Cholesterol and EGF Binding

FIG. 3. Cholesterol loading decreases EGF binding. A, HeLa cells were cholesterol-loaded with the indicated concentration of an mβCD-cholesterol complex for 30 min at 37 °C or left as control. Cells were lysed, and cholesterol and protein concentrations were determined as described under “Experimental Procedures.” Values are given as μg of cholesterol/mg of protein. B, cells were cholesterol-loaded as in A and subsequently incubated with 1 nM 125I-EGF on ice for 1 h, washed, and lysed in 1 M NaOH. All samples were counted in a γ-counter. Values are given as percentage of control. C, cells grown on chamber slides were cholesterol-depleted or cholesterol-loaded for 30 min at 37 °C with the indicated concentrations of mβCD or mβCD-cholesterol or left as control cells and fixed, and EGF receptors on the surface of the unpermeabilized cells were immunolabeled. The average cellular fluorescence intensity, corresponding to the amount of EGF receptors on the surface, was determined by confocal microscopy as described under “Experimental Procedures.” A and B show means ± S.D. from three independent experiments. C shows means ± S.D. of the average cellular fluorescence determined for 8–10 groups of 5–15 cells. *, different from control, p < 0.05; **, different from control, p < 0.01.

FIG. 4. The EGF receptor co-localizes with GM1. A–F, HeLa cells grown on chamber slides were incubated with fluorescent CT-B on ice for 1 h to label GM1 (E), fixed, and stained for caveolin-1 (B) and EGF receptor (A and D). G–I, GM1 (H) was patched by incubating HeLa cells with fluorescent CT-B for 1 h at 12 °C followed by incubation with anti-cholera toxin for 1 h at 12 °C, with washes between incubations. The cells were subsequently fixed and labeled for EGF receptor (G). D–F). Although some of these GM1-containing membrane domains appear to be caveolae, by far the majority of EGF receptors is associated with non-caveolar, GM1-containing membrane domains.

To confirm further that the EGF receptor is indeed localized in GM1-containing membrane domains, we labeled GM1 in HeLa cells by incubating the cells at 12 °C with fluorescent CT-B, and we subsequently induced patching by incubation at 12 °C with anti-cholera toxin antibody. The patched cells were fixed and stained with anti-EGF receptor antibody. As can be seen from Fig. 4H, this treatment clearly induced patching of GM1. Interestingly, the EGF receptor was co-patched with GM1 (Fig. 4, G–I), showing that there is a physical association of GM1 and EGF receptors, which is strong enough to tolerate patching of GM1.

The EGF Receptor Is Localized in Brij 58 Detergent-resistant, Cholesterol-based Membrane Domains—Because Triton X-100 insolubility at 4 °C is an established characteristic of many cholesterol-sphingolipid-containing membrane domains, including both caveolae and non-caveolar lipid rafts (3), we next investigated whether the EGF receptor is associated with Triton X-100-insoluble membrane domains. HeLa cells were lysed in 0.5% Triton X-100, and the lysates were fractionated into supernatants and pellets by centrifugation at 16,000 × g and analyzed by SDS-PAGE followed by immunoblotting. Although caveolin-1 was largely insoluble in Triton X-100, the EGF receptor was completely soluble after centrifugation at 16,000 × g for 15 min (Fig. 5A, 1st and 2nd lanes).

However, complete solubility of a protein in cold Triton X-100 does not exclude that the protein is associated with other Triton X-100-soluble, cholesterol-rich membrane domains or that the interaction of the protein with Triton X-100-insoluble domains is too weak to persist during Triton X-100 extraction. Indeed, this has been shown to be the case for the membrane protein Prominin, which is Triton X-100-soluble but insoluble in other non-ionic detergents, including Brij 58 and Lubrol WX (21). In case of the T-cell antigen receptor, the receptor has been shown by fluorescence microscopy to be associated with GM1-containing lipid rafts, although this interaction is not preserved during Triton X-100 extraction (6). We therefore investigated the solubility of the EGF receptor in the non-ionic detergents Brij 56 and Brij 58. Brij 58 is a detergent with a higher hydrophilic-lipophilic balance than Triton X-100, whereas Brij 56 resembles Triton X-100 (21). HeLa cells were lysed at 4 °C in 0.5% Brij 56 or Brij 58, fractionated into supernatant and pellet by centrifugation, and analyzed by SDS-PAGE followed by immunoblotting for EGF receptor and caveolin. Interestingly, after lysis with Brij 56, some EGF receptor was found in the pellet (Fig. 5A, top panel, lanes 3 and 4), and after lysis with Brij 58 a substantial amount of EGF receptor was found in the pellet (Fig. 5A, top panel, 5th and 6th lanes). When compared with the total amount of protein in the
The EGF receptor is associated with Brij 58 detergent-resistant, cholesterol-based membrane domains. A, HeLa cells were lysed in 0.5% of the indicated detergent for 30 min at 4 °C, the postnuclear lysates fractionated into pellets (P) and supernatants (S) by centrifugation at 16,000 × g for 20 min, and analyzed by Western blotting. B, HeLa cells were cholesterol-depleted with the indicated concentrations of mβCD for 30 min at 37 °C and subsequently lysed in 0.5% Brij 58 and treated as described in A. C, densitometry of B showing the distribution of EGF receptor in supernatant relative to pellet for control and cholesterol-depleted cells.

supernatant and in the pellet as determined by Coomassie Blue staining (not shown), the EGF receptor was highly enriched in the Brij 58 pellet. Caveolin-1 was found in the pellet after lysis with both Brij 56 and Brij 58 (Fig. 5A, bottom panel, 3rd to 6th lanes). Detergent insolubility of a protein may be a result of either localization of the protein to insoluble membrane domains or due to insolubility of the protein itself in the detergent. Therefore, our results showing that the EGF receptor is highly insoluble in Brij 58 do not necessarily mean that the receptor is localized in insoluble membrane domains. However, the Brij 58 insolubility of the EGF receptor together with our immunofluorescence results showing that the EGF receptor co-localizes with the raft marker GM1 strongly support the idea that the receptor is present in GM1-containing, Brij 58-insoluble lipid rafts.

If the association of EGF receptor with Brij 58-insoluble membrane domains is cholesterol-dependent, cholesterol depletion of the cells should cause a shift in the localization of the EGF receptor, from the insoluble membrane fractions in the pellet to the supernatant. To investigate the cholesterol dependence of the association of EGF receptor with Brij 58-insoluble membrane domains, cells were cholesterol-depleted with 5 or 10 mM mβCD for 30 min at 37 °C followed by lysis in cold Brij 58, fractionation into pellet and supernatant by centrifugation, and then analyzed by SDS-PAGE and immunoblotting. Following cholesterol depletion, a clear shift of the EGF receptor toward the supernatant was seen (Fig. 5, B and C), revealing that the Brij 58-insoluble membrane domain containing the EGF receptor is cholesterol-dependent.

We present results showing that the cholesterol level in the plasma membrane regulates EGF receptor ligand binding. Cholesterol depletion increases EGF binding, whereas cholesterol loading decreases EGF binding. The changes in EGF binding are caused by alterations of the number of available EGF-binding sites, B\textsubscript{max}, whereas K\textsubscript{d} values remain unchanged. The changes in B\textsubscript{max} values are not due to altered endo- or exocytosis of receptors but are caused by effects on receptors already present in the plasma membrane. We also show that the EGF receptor is localized in non-caveolar, GM1-containing lipid rafts. These rafts are sensitive to extraction with Triton X-100 but not to extraction with Brij 58. Furthermore, the association of EGF receptors with these rafts is cholesterol-dependent.

Similarly to the data shown here, it has been demonstrated previously (22) that cleavage of cellular gangliosides by sialidase increases EGF binding by increasing B\textsubscript{max}, whereas addition of exogenous gangliosides inhibits EGF binding by lowering B\textsubscript{max}. We therefore propose that the effects of cholesterol and gangliosides on EGF binding are caused by alterations of glycosphingolipid- and cholesterol-rich, non-caveolar membrane domains where the EGF receptor resides. The effect of rafts on ligand binding could be due to direct interactions between the receptor transmembrane domain and raft lipids such as cholesterol and/or glycosphingolipids, or it could be due to raft-facilitated protein-protein interactions. Interestingly, it has been shown recently (23) that the extracellular domain of the EGF receptor interacts with glycosphingolipids, in particular with the ganglioside GM3. However, this interaction does not affect EGF binding to a recombinant form of the extracellular domain in vitro. Whether direct interactions with GM3 affect binding of EGF to the full-length EGF receptor remains to be determined.
Previous studies (1, 4) have shown that in unstimulated cells, the EGF receptor is present in caveolae. These studies are based on density centrifugations of cells fractionated by a detergent-free method. However, although density centrifugations give information about the densities of the membrane domains containing the proteins of interest, they do not give information about whether these proteins are actually present in the same membrane structures or in different structures with the same density. Therefore, the results should be confirmed by other methods such as co-immunoprecipitation studies or morphological studies. We show by immunofluorescence microscopy experiments that the EGF receptor is largely membrane domains. However, our immunofluorescence microscopy experiments show that the EGF receptor was absent from caveolae. This is in agreement with a study in which the behavior of caveolin-1 and the EGF receptor during Triton X-100 extraction (6). It is therefore uncertain whether the differential insolubility of raft structures but that their interaction with raft lipids or other proteins are differentially sensitive to detergent extraction (21), or that the proteins are localized in the same raft structures that do not have the same sensibility to detergent extraction (6). This may reflect either that the proteins of interest are localized in different raft structures that do not have the same raft environment might serve a role in inhibition of signaling as well. We therefore propose the model depicted in Fig. 6, in which lipid rafts function as negative regulators of EGF receptor signaling by sequestering EGF receptors in a state relatively inaccessible for ligand binding. We tentatively speculate that the EGF receptor under physiological conditions moves in and out of the rafts in a regulated way, thereby providing a simple way of controlling EGF receptor ligand binding and signaling.

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