

Regulation of human EGF receptor by lipids

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Contributed by Kai Simons, April 8, 2011 (sent for review March 10, 2011)

The human epidermal growth factor receptor (EGFR) is a key representative of tyrosine kinase receptors, ubiquitous actors in cell signaling, proliferation, differentiation, and migration. Although the receptor is well-studied, a central issue remains: How does the compositional diversity and functional diversity of the surrounding membrane modulate receptor function? Reconstituting human EGFR into proteoliposomes of well-defined and controlled lipid compositions represents a minimal synthetic approach to systematically address this question. We show that lipid composition has little effect on ligand-binding properties of the EGFR but rather exerts a profound regulatory effect on kinase domain activation. Here, the ganglioside GM3 but not other related lipids strongly inhibited the autophosphorylation of the EGFR kinase domain. This inhibitory action of GM3 was only seen in liposomes compositionally poised to phase separate into coexisting liquid domains. The inhibition by GM3 was released by either removing the neuraminic acid of the GM3 headgroup or by mutating a membrane proximal lysine of EGFR (K642G). Our results demonstrate that GM3 exhibits the potential to regulate the allosteric structural transition from inactive to a signaling EGFR dimer, by preventing the autophosphorylation of the intracellular kinase domain in response to ligand binding.

lipid rafts | glycolipid | allosteric modulator | receptor signaling

Cell membranes are composed of a lipid bilayer, containing proteins that either span the bilayer or interact with the lipids on either side of the two leaflets. Although recent advances in lipid analytics demonstrated that these cell membranes contain hundreds of different lipid species (1, 2), the function of this diversity remains enigmatic. Some membrane proteins contain tightly bound lipids that remain associated with the protein even after detergent solubilization and such tightly bound lipids have been resolved in the atomic structure of membrane proteins (3, 4). Over the last three decades, gangliosides (neuraminic acid-containing glycosphingolipids) have been reported to affect growth factor receptor function but whether the lipids exert their modulating function by direct association with the growth factor receptor or indirectly have been difficult to ascertain (5–7). This uncertainty is reflected by the fact that recent reviews of receptor tyrosine kinase signaling fail to include this body of research (8–10). In general, the issue of how membrane proteins interact with lipids in the bilayer is a neglected area of research.

Here, we address the topic of how gangliosides affect the well-studied human epidermal growth factor receptor (EGFR). Early studies showed that changes in cellular ganglioside GM3 modulated tyrosine kinase activity of the receptor in cells (11–13). The inhibitory effect of GM3 was supported by data showing direct binding of GM3, but not other gangliosides, to the purified EGFR ectodomain (14). Depletion of cholesterol from cells, on the other hand, activated the EGFR (15, 16). Other proteins such as caveolin, a cholesterol-binding protein were reported to modulate these interactions (17). To find out whether gangliosides modulate EGFR activity directly we simplified the experimental system by a synthetic biology approach. Here, reconstitution of human EGFR into proteoliposomes enabled us to follow ligand binding as well as receptor autophosphorylation in a minimal system with defined lipid compositions that either form a uniform liquid-disordered (ld) membrane phase or can

phase separate into coexisting liquid-disordered and liquid-ordered (ld/lo) domains. Our data shows that GM3 strongly inhibits the allosteric transition leading to EGFR autophosphorylation and activation without affecting ligand-binding properties, but does so only in phase-separated ld/lo proteoliposomes.

Results

To address the role of lipids in the activity of the human EGF receptor, we bypassed cellular complexity by reconstituting human EGFR into proteoliposomes with defined lipid compositions (*SI Text*) and employed this minimal system to study the role of the lipid environment on receptor function. We produced the full-length human EGF receptor in ExpresSF+ insect cells and purified the receptor to high purity and homogeneity. We tested different lipid compositions and then focused our work on previously well-characterized ternary lipid mixtures of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, sphingomyelin (18:0) and cholesterol in two different compositions (37.5/37.5/25 and 80/15/5 mol%), the former giving rise to two immiscible fluid membrane phases (ld/lo), whereas the latter forms a single ld membrane phase (18, 19). These two membrane systems could then be supplemented by the addition of gangliosides (0.5 mol%), which were reported previously to have effects on receptor activity (6).

EGF Receptor in Defined Lipid Environment Displays only Low Affinity State. The EGF receptor is reported to have two-site ligand-binding affinities, ranging from pM (high affinity) to nM (low affinity) equilibrium dissociation constants (K_D) (20) with the majority of plasma membrane receptors being in the low affinity state (approximately 90%). Concave-up curvilinear Scatchard plots transformed from saturation binding plots typically reflect this two-site ligand binding. To assess the equilibrium dissociation constants in our proteoliposomes, we designed a chemiluminescence assay to measure ligand binding with extremely high sensitivity and broad linear range of detection. In all lipid compositions, we detected only one affinity population of EGF receptor (Fig. 1). The equilibrium dissociation constants of 2–5 nM are in agreement with the affinity range of the low affinity receptor population measured in cultured cells (20). We did not detect a major effect on EGF binding by changing the membrane phase properties of the bilayer or by the presence of gangliosides (*SI Text*), as previously reported (11).

Membrane Heterogeneity Prevents Ligand-Independent Autophosphorylation. To measure autophosphorylation of the EGF receptor kinase domain in proteoliposomes we adapted a previously described protocol for in vitro EGF receptor autophosphorylation in plasma membrane-derived vesicles (15). To allow MgATP

Author contributions: Ü.C., M.G., and K.S. designed research; Ü.C. and M.G. performed research; D.D. contributed new reagents/analytic tools; Ü.C. and M.G. analyzed data; and Ü.C., M.G., and K.S. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1105666108/-DCSupplemental.

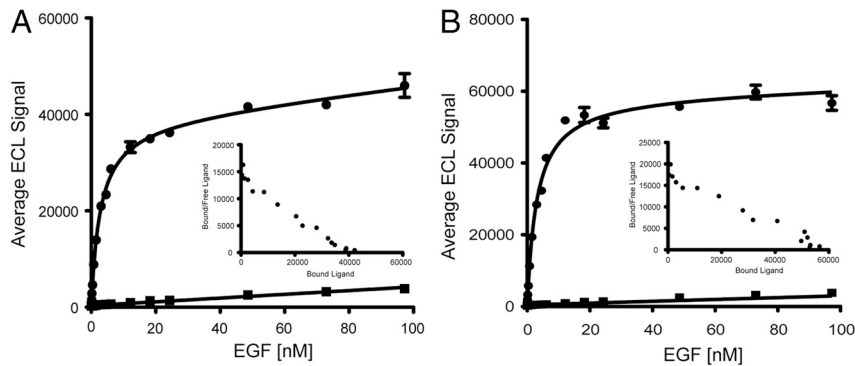


Fig. 1. Full-length EGF receptor reconstituted in proteoliposomes shows only low affinity binding of EGF. Representative saturation plots for Ld (A) and Ld/lo proteoliposomes (B) before background subtraction. Squares show background binding to liposomes. The assayed ligand concentration range of the respective measurement was between 24 pM to 97 nM. Insets represent Scatchard transformation of the respective saturation binding curves after background subtraction. The measured K_D values for EGF binding were in the range of 2–5 nM for all lipid compositions ($n > 5$) (see *SI Text*), corresponding to low affinity state EGF binding. Saturation binding data was analyzed by nonlinear curve fitting using GraphPad Prism 5.0 software.

to reach the cytoplasmic domain of the EGFR in the interior of the liposomes (thereby enabling autophosphorylation), holes were formed in the proteoliposome bilayer by addition of $MnCl_2$ and $RbCl$. We first measured the autophosphorylation profile of Ld/lo versus Ld proteoliposomes. Strikingly, the addition of $MgATP$ in the absence of EGF induced autophosphorylation of the EGF receptor in Ld proteoliposomes, but not in Ld/lo-proteoliposomes (Fig. 2). In phase-separated Ld/lo proteoliposomes, autophosphorylation could be stimulated only by the addition of

EGF, with saturating ligand concentrations maximally stimulating autophosphorylation activity. We therefore concluded that the phase-separating lipid bilayer mixture themselves can act as an attenuator, preventing the receptor from uncontrolled signaling in the absence of ligand.

GM3: Allosteric Inhibitor of EGFR Autophosphorylation. We further analyzed the effect of gangliosides on autophosphorylation. The presence of GM3 in Ld/lo proteoliposomes led to a full in-

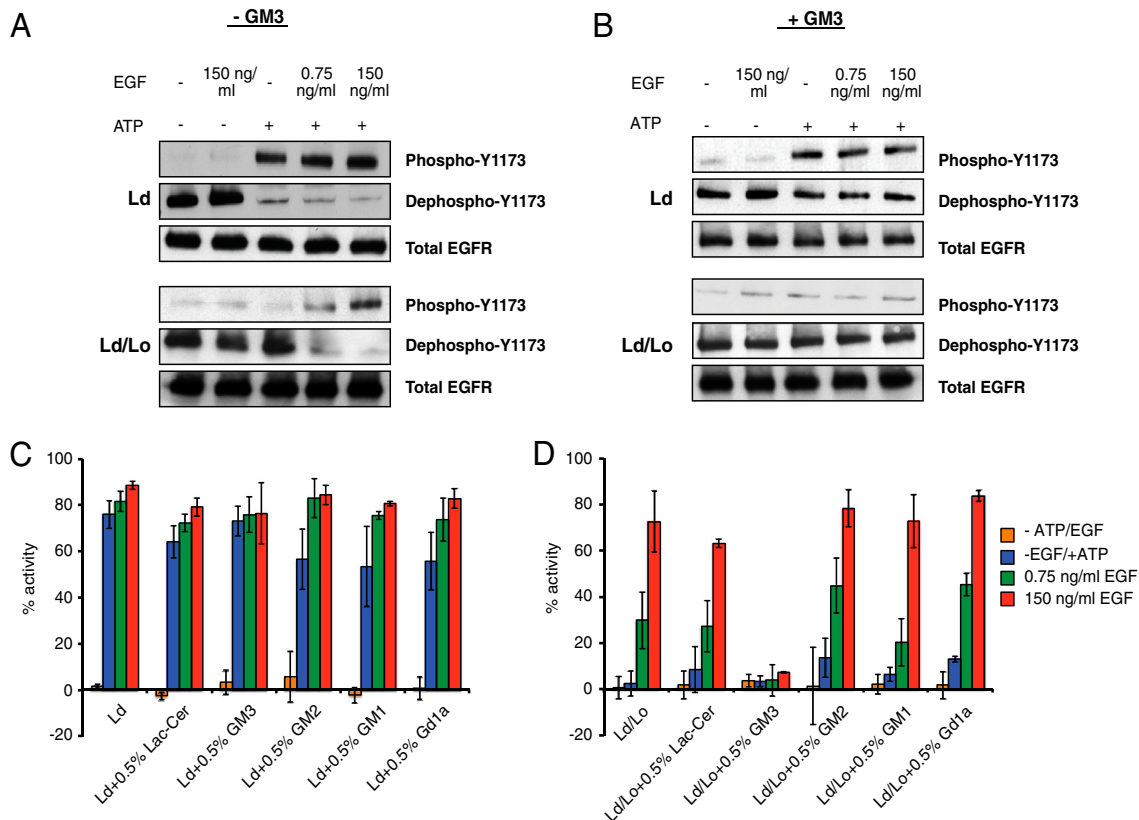


Fig. 2. GM3 inhibits activity of EGF receptor in Ld/lo proteoliposomes. The activity of EGF receptor reconstituted in various lipid environments was measured by antibody detection of transphosphorylated Y1173. Proteoliposomes were incubated in the presence of ligand (0.75 and 150 ng/mL) and ATP (0.25 mM). The activity of the kinase domain was measured by detection of the phosphorylation of Y1173 by two antibodies mapping the same residue in either phospho- or dephospho- state. Representative blots for phosphorylation of EGF receptor are shown for Ld (A) or Ld/lo (B) liposomes. (C) The activity of the receptor in Ld proteoliposomes is not affected by addition of glycolipids, whereas (D) addition of 0.5 mol% GM3 in Ld/lo proteoliposomes leads to total inactivation of EGF receptor activity. Other glycolipids had no effect. Error bars represent SD values. GM3-Ld/lo phosphorylation was assayed with ten independent proteoliposomes preparations. All other conditioned were assayed $n > 3$. Representative Western blots of all lipid compositions can be seen in *SI Text*. Western blots were analyzed using AIDA software (Raytest).

activation of EGFR autophosphorylation, even at saturating EGF concentrations (Fig. 3B). Surprisingly, GM3 had no effect in *ld* proteoliposomes, demonstrating that the phase properties of the membrane significantly contributed to the effect of GM3 on EGFR autophosphorylation. The other gangliosides that we tested (GM2, GM1, and GD1a) and the GM3 precursor lactosylceramide (LacCer) did not affect EGFR autophosphorylation, showing the lipid specificity of the inhibition (Fig. 2). Thus, our data clearly demonstrate that GM3 is capable of inhibiting EGF receptor autophosphorylation, presumably by direct binding to the ectodomain (14).

To gain further insight into the GM3/EGFR binding mechanism, we incubated GM3 containing proteoliposomes with a soluble neuraminidase to convert GM3 to its precursor, lactosylceramide (Fig. 3A and B), which could not inhibit EGFR kinase activity when added into proteoliposomes (Fig. 2). This conversion resulted in full rescue of EGF receptor autophosphorylation, clearly showing that the neuraminic acid of GM3 is involved in EGF receptor interactions. Moreover, under these conditions the neuraminidase should only remove the neuraminic acid from the outer leaflet of the bilayer. Thus the proteoliposomes are functionally asymmetric.

Membrane Proximal K642 Is Involved in EGFR:GM3 Interaction. Analogous to EGFR, the insulin receptor has been reported to be modulated by GM3 binding. Mice lacking GM3 synthesis show increased insulin sensitivity (21), whereas increased levels of GM3 (as in Type I Gaucher disease) impair insulin signaling, resulting in insulin resistance (22). A basic lysine (K944) residue localized extracellularly close to the transmembrane domain of the insulin receptor was shown to be involved in GM3 interaction (23). The amino acid sequence of the EGFR revealed a similarly located membrane proximal lysine (K642) in the ectodomain of the EGFR. To determine whether K642 plays a role in EGFR/GM3 interaction we expressed and purified an EGFR with lysine642 mutated to glycine (EGFR-K642G). EGF binding of the K642G mutant receptor in our proteoliposomal system was at parity with the wild-type receptor (Fig. 4C). However, the lack of the membrane proximal lysine residue resulted in loss of GM3 sensitivity, as evidenced by autophosphorylation activity of the EGFR-K642G kinase domain in the presence of GM3 (Fig. 4D).

GM3 Retains EGFR in the Monomeric State. Chemical cross-linking studies have indicated that GM3 inhibits receptor dimerization in the plasma membrane (12), essential for EGFR activation (24). Using the cross-linker BS³ in our proteoliposomes, we showed that GM3 had no effect on receptor dimerization in the absence of ligand in *ld* proteoliposomes. In contrast, in phase-separated proteoliposomes GM3 prevented the formation of cross-linked dimers (Fig. 5) but EGF addition overcame the inhibition. Thus, GM3 in phase-separated proteoliposomes seemed to stabilize the monomeric form of EGFR.

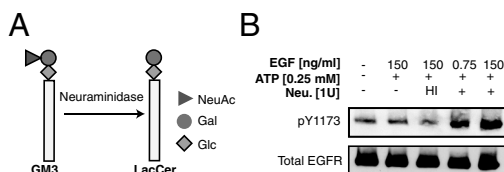


Fig. 3. Neuraminidase treatment of GM3-*ld/lo* proteoliposomes rescues EGF receptor autophosphorylation. (A) The *ld/lo* proteoliposomes containing GM3 were incubated with neuraminidase (30 min, 37 °C), which hydrolyses NeuAc from the headgroup of GM3 converting it to Lac-Cer. (B) HI (heat inactivated) neuraminidase has no effect on the inhibitory effect of GM3 in *ld/lo* proteoliposomes. The presence of active neuraminidase results in full rescue of the WT-EGFR autophosphorylation. In total three independent experiments have been performed.

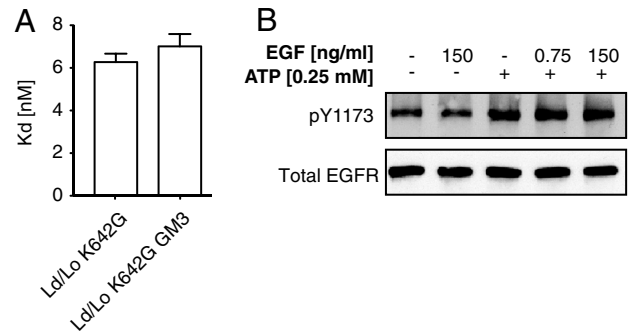


Fig. 4. Mutation of membrane proximal K642 leads to GM3 insensitivity. K_D values of EGFR-K642G mutant receptor *ld/lo* ± GM3 proteoliposomes is not affected to a great extent compared to WT-EGFR (SI Text). Displayed K_D values correspond to a same proteoliposome batch used for the autophosphorylation assay shown in (B). Error bars correspond to the standard error of the mean of duplicate saturation binding measurements. Three independent ligand-binding assays have been performed in total. (B) EGFR-K642G mutation results in an insensitivity with respect to the inhibitory effect of GM3 on EGFR autophosphorylation.

Discussion

The most important finding of this study is that EGFR activity can be regulated by its lipid environment and in this context is specifically inhibited by interaction with the ganglioside GM3. Previous studies have suggested this possibility (6), but because of cellular complexity and membrane compositional diversity, unambiguous assignment of lipid-mediated EGFR modulation has not found its way into the signal transduction canon (8–10). Our findings using purified EGFR reconstituted into proteoliposomes of specific lipid compositions unequivocally demonstrate that whereas the lipid environment does not affect EGF binding, interactions between the receptor and membrane lipids lead to changes in EGFR tyrosine kinase function. A three-component lipid mixture consisting of unsaturated PC, sphingomyelin, and cholesterol in molar ratios that phase separate into coexisting *ld* and *lo* domains prevented EGFR autophosphorylation in the absence of EGF while allowing ligand-mediated receptor dimerization and activation. When GM3 was added to the *ld/lo* proteoliposomes EGFR autophosphorylation was inhibited (Fig. 2) without affecting ligand binding (Fig. 1 and SI Text). The observation that removal of neuraminic acid from GM3 by neuraminidase and the mutation of a single membrane proximal lysine residue rescued EGFR autophosphorylation (Fig. 3) demonstrated the specificity of the lipid–receptor interaction. Our data concerning the head group specificity of the ganglioside are con-

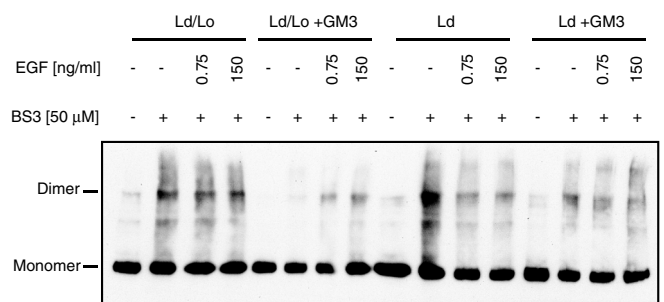


Fig. 5. Ligand-induced dimerization of EGF receptor in proteoliposomes. Prior to cross-linking, EGFR proteoliposomes were incubated with EGF (30 min, RT). For chemical cross-linking BS³ was used (50 μM, 15 min, RT). In the absence of ligand, a significant fraction of the EGFR appears as preformed dimers. In *ld/lo* proteoliposomes the presence of GM3 is accompanied by a significant decrease of this preformed dimer fraction whereas ligand driven dimerization is not affected. Molecular weight determination is shown in SI Text. In *ld* proteoliposomes GM3 has no effect in EGFR dimer formation. In total four independent experiments have been performed.

sistent with previous binding studies based on the purified ectodomain of EGFR where immobilized GM3 could bind soluble ectodomain (14).

One issue that has occupied the EGFR field for decades has been the characteristic concave-up curvilinear Scatchard plots of EGF binding (25, 26). These plots have been interpreted to signify heterogeneity of ligand-receptor binding. Recent structural studies on the ectodomain of the *Drosophila* EGF receptor proposed a negative cooperativity in ligand binding (27). Upon binding of the *Drosophila* EGF-like domain of Spitz (Spitz_{EGF}) to the *Drosophila* EGFR, the first ligand-binding event induced an asymmetric dimer with only one Spitz_{EGF} bound. The structural data suggested that the unoccupied site on the second EGFR subunit was restrained by the first binding event, leading to reduced binding affinity for the second Spitz_{EGF} molecule in the asymmetric dimer. However, the isolated human EGFR ectodomain does not form asymmetric dimers like its *Drosophila* counterpart (27, 28). Thus if the negative cooperativity were to explain heterogeneity in ligand binding, the intracellular part of the EGFR would be driving the formation of an asymmetric dimer as previously suggested (29). Our binding studies showed that the full-length human EGFR reconstituted into liposomes failed to display high affinity binding. Although our cross-linking data suggested the presence of preformed dimers (Fig. 5), we observed only one affinity state of the EGFR (Fig. 1 and *SI Text*). Similarly, previous studies have shown that EGFR in isolated plasma membrane vesicles also display only low affinity binding, although the cells from which plasma membranes were isolated exhibited both EGF-binding states (30). A recent study on live cells demonstrated the preferential presence of high affinity dimers in the cell periphery in an actin- and receptor expression-dependent fashion (31). Together with our data, these findings suggest that interactions with additional intracellular factors are required to invoke high affinity sites, as postulated previously (20).

An unresolved issue is how the lipid environment could influence EGFR activity. Whereas the structure of full-length EGFR has not yet been solved, structural studies of the EGFR ectodomain and the cytoplasmic tyrosine kinase domain have identified four distinct structural states: an autoinhibited tethered monomer, an extended monomer, an inactive symmetric dimer and an active asymmetric dimer (32–34). Ligand binding to the tethered monomer promotes the release of the inhibitory tether and formation of an extended monomer, necessary for the formation of a stable dimer (33, 35). Recent studies identified ligand-independent, non-signaling preformed dimers (16, 31, 34, 36, 37), where the formation of the active dimer structure is guided by an intracellular juxtamembrane segment (34, 38, 39) as well as interactions between the EGFR transmembrane domains (34). The inactive tethered monomers and the symmetric inactive dimer interconvert in a steady-state equilibrium on the cell surface. To generate the asymmetric active dimer, the C-terminal ends of the ectodomains have to be brought close together at the junction with the membrane where the transmembrane segments descend into the bilayer. In the absence of ligand, the extracellular domains of the EGFR hinder the formation of the active dimer. Jura et al. postulated that the extracellular domain and the inactive dimer work synergistically to prevent the high local concentration of receptors at the membrane from generating spurious signals through transphosphorylation (34). The question now becomes how this balance is regulated. Our results suggest that the lipid environment of the receptor may play a role in inhibiting the formation of active dimers in ligand-unbound receptors. Our cross-linking studies suggest that inclusion of GM3 in phase-separating liposomes, promotes a significant shift in the balance from dimers to monomers in the absence of EGF (Fig. 5). In this manner, GM3 accentuates the auto-inhibitory mechanism of the ligand-unbound EGFR.

Another important aspect of EGFR regulation by membrane lipids is that EGFR activation is stimulated by cholesterol depletion of plasma membranes (15, 16). This is in agreement with our results showing that the cholesterol-poor ld proteoliposomes allow EGFR autophosphorylation, whereas cholesterol-rich ld/lo proteoliposomes promote EGFR auto-inhibition (Fig. 2). How these findings correspond to EGFR behavior in living cells is not clear. One possibility is that inactive, tethered monomers of EGFR could preferentially associate in a dynamic fashion with nanoscale lipid rafts (40). EGF binding would dissociate EGFR from the raft assembly to enable dimer formation and activation. GM3 binding to the EGFR ectodomain could further promote or stabilize the inactive raft-conformation. We therefore suggest that GM3:EGFR ectodomain interaction might additionally perturb the intrinsic capacity of transmembrane- and juxtamembrane-dimerization (34), preventing activation of the kinase domains via asymmetric dimerization. It will be interesting to see whether a GM3–EGFR complex can be crystallized and its structure be resolved.

Our study thus clearly shows that lipids can function in the allosteric regulation of EGFR function. Previous work suggests that such lipid-protein interactions could be much more widespread than generally realized. Other receptor tyrosine kinases such as FGF-, Trk A-, PDGF-, and insulin receptor have been reported to be modulated by specific gangliosides (6). Glycosphingolipids are a large family of molecules in search of functions and our results, together with previous clues alluding to their role in signaling, necessitate follow-up by biochemical and structural studies.

Materials and Methods

EGF-Binding Assay. EGF-biotin was conjugated with Sulfo-Tag TM (STAG) labeled streptavidin (Streptavidin-STAG, Mesoscale Discovery) (MesoScale in 1:3 molar ratio and incubated on ice for 2 h. Proteoliposomes were passively adsorbed on the electrode surface (1 h, 23 °C), the residual sites on the surface were blocked with 0.1% bovine gamma globulin (DB-blocker, MesoScale Discovery) (1 h, 23 °C). The surface was then washed three times with Hepes liposome buffer (25 mM Hepes, 150 mM NaCl, pH 7.25). Ligand binding was initiated by addition of EGF-biotin–Streptavidin–STAG complex in the desired dilution range and incubation for 2 h at 23 °C. Wells were then washed and reading buffer was added (surfactant-free reading buffer, MesoScale Discovery). All data points were collected as duplicates. Background was determined from binding of EGF-biotin–Streptavidin–STAG to empty liposomes. Data were acquired on a SECTOR Imager 6000 chemiluminescence reader. Saturation binding data were analyzed by nonlinear curve fitting using GraphPad Prism 5.0 software.

Phosphorylation Assay. For EGFR phosphorylation assay a modified protocol from Pike and Casey was used (15). EGF was added to the proteoliposomes and incubated for 2 h (RT). All chemical stocks were prepared freshly. Assays were started after addition of reaction mix (final assay concentration—12 mM MgCl₂, 2 mM MnCl₂, 2 mM RbCl, 100 mM NaVO₄, 20 mM para-Nitrophenylphosphate (PNPP), 20 mM β-glycerophosphate, 0.25 mM ATP) and the samples were incubated for 30 min (30 °C, 600 rpm). The reaction was stopped by addition of denaturing SDS sample buffer followed by SDS-PAGE. For the neuraminidase rescue experiment, the enzyme (*Clostridium perfringens* Neuraminidase, New England Biolabs) was added to the samples during the last 30 min incubation with EGF.

ACKNOWLEDGMENTS. The authors thank all past and present members of the Simons laboratory. We thank Anna Shevchenko and Julio Sampaio for their skillful mass spectrometric analysis of our samples. We also thank Li Ying (Nanyang Technological University, Singapore) for help with the initial ligand-binding setup and Robert Umek [Mesoscale Discovery (MSD)] for continues technical support with the MSD chemiluminescence platform. This work was supported by the following agencies: Deutsche Forschungsgemeinschaft (DFG) "Schwerpunktprogramm 1175" Grant SI459/2-1 and "Transregio 83" Grant TRR83 TP02, European Science Foundation (ESF) "LIPIDPROD" Grant SI459/3-1, Bundesministerium für Bildung und Forschung (BMBF) "ForMat" Grant 03FO1212, and The Klaus Tschira Foundation.

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