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Previous studies have shown that ~60% of the epidermal growth factor receptors (EGFRs) in quiescent fibroblasts are concentrated in the caveolae/raft fraction from purified plasma membranes. This high degree of localization suggests the EGFR contains targeting information for lipid domains. We have used mutagenesis to determine that the region of the receptor that controls targeting to caveolae/rafts resides in the juxtamembrane, second cysteine-rich region. A 60-amino acid-long sequence within this region that is continuous with the transmembrane domain was sufficient to target the transmembrane and cytoplasmic tails of both EGFR and the low density lipoprotein receptor to caveolae/rafts. Two *N*-linked sugars in this segment were not required for proper targeting, although unglycosylated wild-type receptors did not localize properly. We conclude that, in contrast to signals for coated pit localization that are in the cytoplasmic tail, the targeting information for caveolae/rafts is on the extracellular side of the EGFR very close to the membrane.

Epidermal growth factor receptors are type I receptor tyrosine kinases that contain an *N*-linked glycosylated extracellular ligand binding domain, a single transmembrane-spanning domain, and an intrinsic cytoplasmic protein tyrosine kinase (1). Members of this family include EGFR/ErbB1 (HER1), ErbB2 (Neu or HER2), ErbB3 (HER3), and ErbB4 (HER4). These receptors are highly conserved during evolution and function in various cellular signaling processes that include cell proliferation, adhesion, migration, differentiation, survival, and apoptosis (1–4). Prior to ligand binding to EGFR[1] in quiescent fibroblasts, a significant portion (65%) of the receptor is in the low density plasma membrane fractions that contain caveolae and non-caveolae rafts (5). After ligand binding, activated receptors rapidly move from this membrane fraction to bulk plasma membrane where they are internalized by clathrin-coated pits (1, 6, 7). Movement to the non-raft fraction is dependent on an active kinase domain in the receptor, the presence of at least one tyrosine residue in the regulatory region of the cytoplasmic region, and an unphosphorylated threonine 654 (5).

Whereas the motifs for receptor internalization and degradation appear to be located in the cytoplasmic portion of EGFR (6), receptors lacking the entire cytoplasmic region still concentrate in the caveola/raft fraction (5). This suggests that either the transmembrane domain or the extracellular region of EGFR contains the targeting information for caveolae/rafts. We have used genetic engineering to distinguish between these two possibilities. Mutant EGF receptors were genetically engineered (Fig. 1), inserted into mammalian expression vector, and transiently expressed in B82 mouse fibroblasts that lack endogenous EGFR. The distribution of the various constructs on the plasma membrane was then quantified using cell fractionation. We were able to localize the principle caveola/raft targeting information in EGFR to a 60-amino acid-long (residues 581–641) juxtamembrane region in the extracellular portion of the receptor. Attachment of this sequence to the transmembrane and cytoplasmic tail of either the EGFR or the LDLR was sufficient to target the molecule to the caveola/raft fraction.

EXPERIMENTAL PROCEDURES

Materials

Human EGFR cDNA and B82 cell lines were kindly provided by Dr. Gordon Gill. High fidelity DNA polymerase (*Pfu*) was purchased from Invitrogen. T4 DNA ligase and all restriction enzymes were purchased from New England Biolabs (Beverly, MA). Mammalian expression vector pcDNA3.1 and pcDNA3.1+Myc-His were from CLONTECH. Plasmid preparation kits and transfection reagents (Superfect) were from Qiagen (Valencia, CA). Low glucose DMEM and Optiprep were from Invitrogen. Fetal bovine serum was purchased from Atlanta Biologicals (Atlanta, GA). Percoll was purchased from Amersham Biosciences. A protease inhibitor mixture was from Roche Molecular Biochemicals. α-EGFR pAb was from Santa Cruz Biotechnology (Santa Cruz, CA). α-Caveolin-1 pAb was from BD PharMingen. α-Myc pAb was from Upstate Biotechnology (Waltham, MA). α-LDLR pAb was kindly provided by Dr. Joachim Herz. All reagents for SDS-PAGE and Western blot analysis were from Bio-Rad. Polyvinylidene difluoride membrane was from Millipore (Bedford, MA). Enhanced chemiluminescence (ECL) reagent was from Amersham Biosciences. The FluorChem 8000 was from Alpha Innotech Corp. (San Leandro, CA).

Methods

Construction of Mutant EGF Receptors—A novel technique was developed to produce the EGFR constructs needed for this study. Instead of using restriction sites to cut and join DNA in the EGFR cDNA, compatible cohesive ends at the desired ligation sites were generated using PCR. For each site, two sets of oligonucleotides were designed for generating sticky ends at the site where the mutation was to be introduced. Two pieces of DNA were amplified from these two sets of oligonucleotides and from the primers at either the 5′-end (containing *Kpn*I sites) or the 3′-end (containing *Xba*I sites) of EGFR cDNA. The DNA products were mixed together, denatured, and reannealed. One-quarter of the reannealed products corresponded to cDNA inserts with the correct compatible ends. DNA products were then cut with *Kpn*I and *Xba*I and ligated together with linearized pcDNA3.1+ expression vector using standard ligation methods. To generate Myc-tagged constructs, the cDNAs were cloned into *Kpn*I and *Xba*I sites of a pcDNA3.1+Myc-His expression vector. Automated DNA sequencing

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[1] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; LDL, low density lipoprotein; LDLR, LDL receptor; DMEM, Dulbecco's modified Eagle's medium; pAb, polyclonal antibody; ECL, enhanced chemiluminescence; 3-D, three-dimensional.

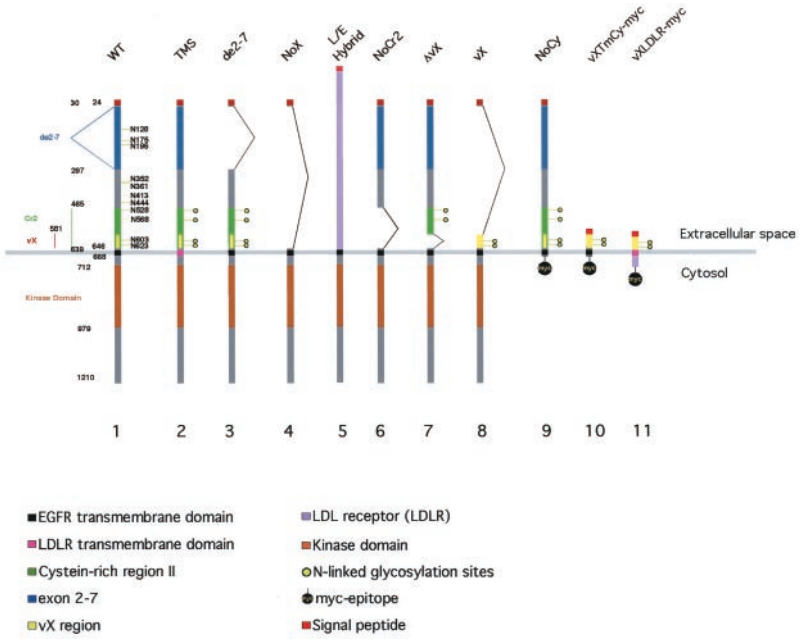

FIG. 1. **Diagrammatic representation of the constructs used in this study.** The various parts of the receptor are indicated by the *color code*. *Construct 1*, the wild-type EGFR (*WT*); *construct 2*, LDL receptor transmembrane domain in place of EGFR transmembrane domain (*TMS*); *construct 3*, EGFR de2-7; *construct 4*, no extracellular region (*NoX*); *construct 5*, extracellular domain of LDL receptor (*L/E Hybrid*); *construct 6*, no second cysteine-rich region (*NoCr2*); *construct 7*, no vX region (*ΔxV*); *construct 8*, vX region alone; *construct 9*, Myc tag in place of the cytoplasmic tail (*NoCy*); *construct 10*, transmembrane and vX region alone (*vXmCy-myc*); *construct 11*, vX region attached to transmembrane domain and cytoplasmic tail of LDLR (*vXLDLR-myc*).

analysis was used to confirm that each construct was correct. We used this method to generate 11 different cDNAs (Fig. 1).

Cell Culture—Normal human fibroblasts and transformed mouse fibroblasts that lack endogenous EGFR (B82 cell lines) were used in this study. Normal human fibroblasts were obtained by skin biopsy, cultured in a monolayer, and set up according to a standard format. On day 0, 2.5×10^5 cells were seeded into 100-mm dishes with 5 ml of minimum Eagle's medium supplemented with 10% fetal calf serum. The medium was changed on day 3 and day 5. All experiments were carried out on day 8. For detection of LDLR, the cells were grown overnight in DMEM on day 7. B82 cell lines were grown in low glucose DMEM plus 10% fetal bovine serum in a 37 °C incubator containing 5% CO_2. One day prior to transfection, 1×10^6 cells were seeded into 100-mm dishes. Thirty-six hours after transfection, cells were ~90% confluent.

Transfection—DNA was prepared using an endotoxin-free plasmid preparation kit (EndoFree Maxi kit) from Qiagen and transfected into B82 cell lines using Superfect transfection reagent according to the manufacturer's protocol. A ratio of DNA to transfection reagent at 1:10 was used in all experiments. We typically achieved 75–80% transfection efficiency with very little cell death. All experiments were done 36 h after transfection.

Isolation of Caveolae/Rafts—Caveolae/rafts were prepared using the detergent-free method of Smart *et al.* (8) with the following modifications for B82 cells. A plasma membrane fraction was prepared from five 100-mm dishes after 36 h of transfection. A glass tissue grinder (Wheaton, catalog no. 357538) was used instead of a Teflon homogenizer. The isolated plasma membrane was sonicated three successive times at a power setting of 50 J/watt·s.

Quantification of EGFR in Plasma Membrane—EGFR was detected by immunoblotting using an α-EGFR pAb as the primary antibody, horseradish peroxidase-conjugated α-rabbit IgG as a secondary antibody, and ECL to visualize the protein. The ECL image was recorded using the FluorChem 8000, and the optical density of an equal surface area for each band was determined using NIH image version 1.6 software. The total optical density value of the bands in 13 fractions equaled 100%. The percentage of signal from fractions 1 to 7 was calculated as the amount of EGFR in the caveola/raft fractions.

RESULTS

A standard cell fraction assay has been developed that can distinguish whether a protein of interest is in caveolae/rafts (8). The light buoyant density of caveolae/rafts allows one to separate them from bulk membrane once the plasma membrane has been fragmented by sonication. Equal volume loads of each fraction were separated on SDS-PAGE gels and immunoblotted to detect the indicated protein (Fig. 2). Protein determinations (data not shown) and Coomassie Blue staining (Fig. 2, *Protein*) of the transferred gels both showed that most of the membrane

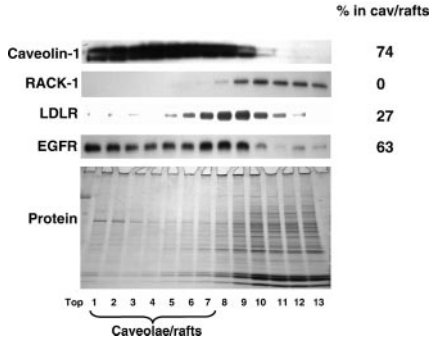

FIG. 2. **Detergent-free separation of caveolae/rafts from bulk plasma membrane.** Normal human fibroblasts were cultured as described. A plasma membrane fraction was isolated from these cells, sonicated, and separated on an Optiprep gradient (8). Equal volumes of each fraction were separated by gel electrophoresis (4–20%) and immunoblotted with an antibody directed against the indicated protein. The density of the immunoblotted band was used to calculate the relative amount of each protein in the caveolae/raft fraction (indicated by *brackets*). After the gel had been transferred, the gel was stained to show the relative amounts of protein in each lane. Much more protein is at the *bottom* of the gradient. *cav*, caveolae; *RACK*, receptor for activated C kinase.

protein was in the dense fractions 8–13. The relative amount of the immunoblotted protein in the caveola/raft fraction (the sum of fractions 1–7) was then quantified by gel densitometry. Using caveolin-1 as a standard marker for caveolae/rafts, on average >74% of this protein was in this fraction (Fig. 2, *Caveolin-1*). By contrast, we found that the receptor for activated C kinase was excluded from the caveolin-rich fraction (Fig. 2, *RACK-1*). Only 27% of the LDLR was in fractions 1–7, but these fractions contained 63% of the EGFR. Importantly the LDLR was confined to a region in the middle of the gradient that overlapped with the densest population of caveolin-rich membranes (fractions 5–7), while the EGFR was enriched throughout fractions 1–7 just like caveolin-1.

Previously we showed that a truncated EGFR containing only a 20-amino acid cytoplasmic segment was enriched in the caveolae/raft fraction (5). We confirmed that the cytoplasmic region is not involved by constructing an EGFR with a Myc tag in place of the normal cytoplasmic tail (Fig. 1, *construct 9*). The fractionation pattern of this EGFR expressed in B82 cells was

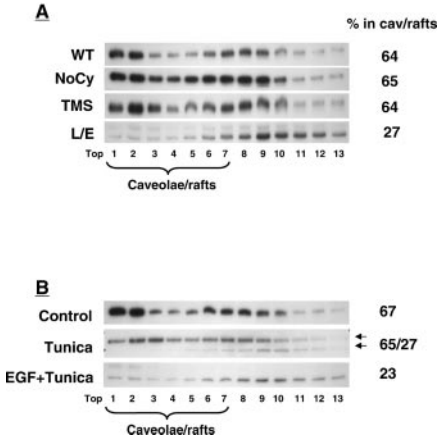

FIG. 3. **The extracellular region of the EGFR contains the targeting information for caveolae/rafts.** *A*, B82 cells were transfected with a cDNA coding for the indicated protein. Cells were grown for 36 h before fractionation of the plasma membrane on Optiprep gradients. *B*, normal human fibroblasts were grown in the presence (*Tunica*) or absence (*Control*) of tunicamycin for 18 h. Alternatively they were first incubated in the presence of 100 ng of EGF for 2 h before being incubated further in the presence of tunicamycin (*EGF + Tunica*). The plasma membrane was fractionated on Optiprep gradients as described. The relative amounts of each protein in the caveolae/rafts are shown on the *right*. Constructs in *A* have been described in Fig. 1. *cav*, caveolae.

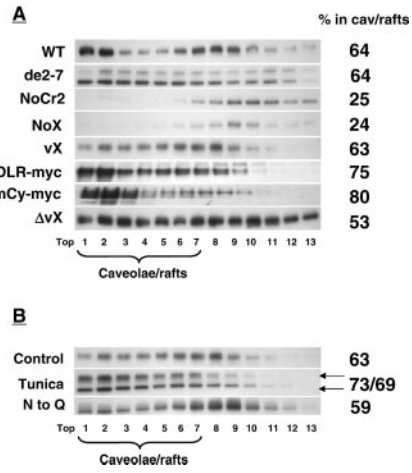

FIG. 4. **The second cysteine-rich region of EGFR contains the sorting information for caveolae/rafts.** B82 cells were transfected with a cDNA coding for the indicated protein and grown for 36 h. *A*, the plasma membrane was fractionated on Optiprep gradients, and the fractions were separated by gel electrophoresis and immunoblotted with antibodies against the indicated protein. *B*, B82 cells were stably transfected with either vX EGFR (*Control* and *Tunica*) or transiently transfected with a vX EGFR that had Asn-603 and Asn-623 changed to glutamine (*N to Q*). One set of cells was incubated an additional 18 h in the presence of tunicamycin (*Tunica*). At the end of the incubations, the plasma membrane was fractionated and immunoblotted as described in Fig. 3. The relative amounts of each protein in the caveolae/rafts are shown on the *right*. Constructs in *A* have been described in Fig. 1. *cav*, caveolae.

identical to the wild-type protein (Fig. 3*A*, compare *WT* and *NoCy*). Therefore, targeting information for caveolae/rafts is either in the transmembrane or the extracellular region of the molecule. To distinguish between these possibilities, we expressed an EGFR that had the transmembrane segment replaced with the one from the LDL receptor (Fig. 1, *construct 1 versus construct 2*). Fractionation of the plasma membrane from these cells showed that swapping the transmembrane region had no effect on targeting the receptor to the caveolae/raft fractions (Fig. 3*A*, compare *WT* with *TMS*). By contrast, swapping the EGFR and LDLR extracellular regions (Fig. 1, *construct 5*) shifted the EGFR hybrid molecule to the bulk membrane fractions (Fig. 3*A*, *L/E*). We conclude that targeting of the EGFR to caveolae/rafts depends on information in the 615-amino acid-long extracellular domain of the receptor.

There are 11 *N*-linked glycosylation sites in the extracellular region of EGFR (Fig. 1, *WT*) that collectively or individually could be involved in EGFR targeting to caveolae/rafts. We used tunicamycin to block glycosylation and then determine the distribution of the unglycosylated receptor in light and heavy membrane fractionations (Fig. 3*B*). Cells were incubated in the presence (*Tunica*) or absence (*Control*) of tunicamycin (4 μg/ml) for 18 h, fractionated, and immunoblotted for EGFR. The EGFR from control cells had a normal fractionation pattern. Tunicamycin-treated cells, by contrast, had an EGFR band with the molecular weight of EGFR (Fig. 3*B*, *upper arrow*) and a lower band that was the right size for unglycosylated EGFR (Fig. 3*B*, *lower arrow*). The lower band was clearly shifted toward the dense membrane fraction relative to the upper band (compare *upper* and *lower arrows*). The turnover of the receptor apparently is too slow to replace completely the glycosylated species during the time course of this experiment. To circumvent this problem, we incubated cells in the presence of EGF to stimulate EGFR degradation before exposing the cells to tunicamycin. Nearly all of the receptors were unglycosylated and shifted toward the more dense, bulk membrane fractions (Fig. 3*B*, *EGF + Tunica*). These results confirm that the extracellular domain directs EGFR to caveolae/rafts and suggests that glycosylation plays a role in the targeting process.

Most likely EGFR clustering in caveolae/rafts depends on a specific amino acid sequence in the extracellular region. To find the location of this sequence, we constructed EGF receptors that were missing specific segments of the molecule (Fig. 1). Previously we showed that an oncogenic form of EGFR (de7-2) that lacks the first 267 amino acids (Fig. 1, *construct 3*) was enriched in caveolae/rafts (5). Fig. 4 shows that B82 cells transfected with cDNA coding for either the wild-type or the de7-2 receptor had the same proportion of receptors in the caveolae/raft fraction (~64%). By contrast, when we removed the 174-amino acid, cysteine-rich region between residues 639 and 465 just above the membrane-spanning region (Fig. 1, *construct 6*), nearly all of the receptors were shifted to the dense membrane fractions (Fig. 4*A*, *NoCr2*). Likewise, removal of the entire extracellular region (Fig. 1, *construct 4*) shifted the truncated receptor into the dense membrane fraction (Fig. 4*A*, *NoX*).

An oncogenic form of the EGFR, v-ErB, is missing the first 580 amino acids (9–11). The remaining 60 extracellular amino acids (residues 581–641) have been designated the vX region. When we expressed the vX EGFR in B82 cells and prepared membrane fractions, its distribution was exactly like the wild-type receptor (Fig. 4*A*, compare *WT* with *vX*). A comparison with the distribution of the truncated (*NoX*) EGFR suggests the vX region is sufficient to target a membrane protein to caveolae/rafts. To determine whether this was correct, we constructed a cDNA coding for a protein that has the vX region attached to the transmembrane and cytoplasmic portions of the LDLR and expressed it in B82 cells (Fig. 1, *construct 11*). Fractionation of the plasma membrane showed that ~75% of the protein was in the caveolae/raft fraction (Fig. 4*A*, *vXLDLR-myc*). The same result was obtained when vX was attached to the transmembrane domain of EGFR (Fig. 4*A*, *vXTmCy-myc*). Curiously, when we constructed a receptor with the vX region deleted (Fig. 1, *construct 7*), the mutant EGFR was equally distributed between heavy and light membrane fractions (Fig. 4*A*, Δ*vX*). We conclude that although the vX region is sufficient for moving the transmembrane and cytoplasmic region of both EGFR and LDLR to caveolae/rafts, additional parts of the second cysteine-rich region may be needed to target the whole receptor.

The vX region contains *N*-linked glycosylation sites at positions 603 and 623. We did two experiments to see whether these positions were part of the targeting machinery. We grew cells stably expressing vX EGFR in the presence (Fig. 4*B*, *Tunica*) or absence (*Control*) of tunicamycin. Unlike the whole receptor (Fig. 3), the lower molecular weight unglycosylated vX EGFR (Fig. 4*B*, *lower arrow*) was concentrated in caveolae/rafts to the same extent as both the glycosylated vX EGFR (Fig. 4*B*, *Tunica*, *upper arrow*) and the vX EGFR from untreated cells (Fig. 4*B*, *Control*). Likewise, changing the two asparagines to glutamine had little effect on the localization of vX EGFR to the caveolae/raft fraction (Fig. 4*B*, *N to Q*).

DISCUSSION

The goal of this study was to identify the region within the EGFR that controls traffic of the receptor to caveolae/rafts in quiescent cells. Just as the cytoplasmic tail of occupied EGFR contains targeting information for coated pits (1), we thought the unoccupied receptor should contain information for targeting to caveolae/rafts. We systematically deleted different regions of EGFR and analyzed the distribution of each mutant protein. In agreement with our previous observations (5), we could not find any requirement for the cytoplasmic tail (Fig. 3*A*). By contrast, removal of the second cysteine-rich region quantitatively shifted EGFR to the bulk membrane fraction, and a 60-amino acid-long sequence within this region, designated vX, was sufficient to shift the transmembrane and cytoplasmic parts of EGFR from the bulk membrane to the caveolae/raft fraction. The vX region was also able to target the LDLR transmembrane and cytoplasmic tail to caveolae/rafts. We conclude that the vX region contains a molecular address for caveolae/rafts.

The membrane fractionation protocol we used in these studies does not use detergents and can only determine whether EGF receptors are in plasma membrane caveolae/rafts. A popular method for isolating caveolae/rafts relies on the intrinsic insolubility of these domains in Triton X-100 at 4 °C (12, 13), but we could not use this method because Triton X-100 extracts EGFR from caveolae/rafts. Detergent insolubility, however, has been used to identify two motifs that appear to be involved in the association of integral membrane proteins with caveolae/rafts during apical sorting from the Golgi apparatus of polarized cells. These are the transmembrane domains of influenza virus hemagglutinin (14) and neuraminidase (15) and the *O*-glycosylation stalk of neurotrophin (16, 17) and sucrase isomaltase (18). These motifs seem not to be involved in the sorting of EGFR to plasma membrane caveolae/rafts because the transmembrane domain of the LDLR could substitute for the EGFR (Fig. 3*A*), and EGFR does not have an *O*-glycosylation stalk. Importantly there is an *O*-glycosylation stalk in the juxtamembrane region of the LDLR extracellular domain, but apparently it cannot substitute for the EGFR extracellular domain (Fig. 3*A*).

N-Glycosylation has also been implicated in raft-dependent apical sorting (19). We found a significant shift of unglycosylated EGFR from caveolae/rafts to the bulk membrane fraction when cells were incubated in the presence of tunicamycin, suggesting that glycosylation is important (Fig. 3*B*). Tunicamycin, however, had no effect on the ability of the vX EGFR construct to collect in caveolae/rafts. Moreover, mutating asparagine 603 and 623 to glutamine had no effect on vX EGFR targeting. The unglycosylated EGF receptors produced in the presence of tunicamycin may be misfolded (20) and, as a result, not targeted properly to caveolae/rafts.

The common theme emerging from these studies is that the information for sorting to caveolae/rafts is not in the cytoplasmic tail of transmembrane receptors like EGFR but instead resides close to the extracellular side of the membrane. Both the vX region in EGFR and the *O*-glycosylation stalk in neurotrophin and sucrase isomaltase are continuous with the extracellular end of the transmembrane domain. Furthermore, the sorting information in the transmembrane domain of hemagglutinin has been localized to that portion in the extracellular half of the membrane (14). The location of this sorting information raises the possibility that the mechanism of molecular capture by caveolae/rafts depends on an interaction between the sorting region of the protein and lipids enriched in the lipid domain. One class of lipids that is enriched is the sphingolipids. A combinatorial extension analysis of a 3-D structural data base has revealed a motif common to β-amyloid and prion, two proteins that are frequently found in caveolae/rafts (21). This motif binds sphingolipids and is located near the membrane insertion site for these proteins. The same motif is also found in the V3 loop of human immunodeficiency virus gp120 where it mediates viral attachment to lipid domains. Although this motif has a similar 3-D structure in these three proteins, the primary sequences are not homologous. Further work will be required to determine whether the vX region binds sphingolipids and has a similar 3-D structure.

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