Cholesterol-Induced Protein Sorting: An Analysis of Energetic Feasibility

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ABSTRACT The mechanism(s) underlying the sorting of integral membrane proteins between the Golgi complex and the plasma membrane remain uncertain because no specific Golgi retention signal has been found. Moreover one can alter a protein's eventual localization simply by altering the length of its transmembrane domain (TMD). M. S. Bretscher and S. Munro (*Science*. 261:1280–1281, 1993) therefore proposed a physical sorting mechanism based on the hydrophobic match between the proteins' TMD and the bilayer thickness, in which cholesterol would regulate protein sorting by increasing the lipid bilayer thickness. In this model, Golgi proteins with short TMDs would be excluded from cholesterol-enriched domains (lipid rafts) that are incorporated into transport vesicles destined for the plasma membrane. Although attractive, this model remains unproven. We therefore evaluated the energetic feasibility of a cholesterol-dependent sorting process using the theory of elastic liquid crystal deformations. We show that the distribution of proteins between cholesterol-enriched and cholesterol-poor bilayer domains can be regulated by cholesterol-induced changes in the bilayer physical properties. Changes in bilayer thickness per se, however, have only a modest effect on sorting; the major effect arises because cholesterol changes also the bilayer material properties, which augments the energetic penalty for incorporating short TMDs into cholesterol-enriched domains. We conclude that cholesterol-induced changes in the bilayer physical properties allow for effective and accurate sorting which will be important generally for protein partitioning between different membrane domains.

INTRODUCTION

Several lines of evidence show that membrane protein sorting between the Golgi complex and the plasma membrane is determined, at least in part, by the length of the proteins' transmembrane domain (TMD). First, Golgi membrane proteins tend to have shorter TMDs (~15 AA) than plasma membrane proteins (~20 AA) (Bretscher and Munro, 1993; Masibay et al., 1993). Second, a protein, that is normally retained in the Golgi complex, becomes targeted to the plasma membrane if the TMD is increased in length (Cole et al., 1998; Masibay et al., 1993; Munro, 1991)—but is minimally affected if the TMD is replaced by a Leu sequence of the same length as the native segment (Munro, 1991). Third, proteins that normally traffic to the plasma membrane are retained in the Golgi complex if the hydrophobic length of the TMD is shortened (Sivasubramanian and Nayak, 1987). Fourth, no specific Golgi retention signal has been identified, and the mechanism underlying the retention of Golgi proteins cannot be saturated by overexpression (Gleeson, 1998; Nilsson and Warren, 1994; Opat et al., 2001). Taken together, these results suggest that the sorting mechanism(s) underlying protein retention in the Golgi complex depend on some general physical characteristic of the bilayer-protein interactions.

Bretscher and Munro (1993) proposed such a physical mechanism, which was based on the following observations: first, cholesterol increases the thickness of lipid bilayers (Nezil and Bloom, 1992); and second, the cholesterol content of the cellular membranes increases along the secretory pathway such that cholesterol in the plasma membrane constitutes ~50% of the membrane lipids (van Meer, 1989). Cholesterol therefore was proposed to regulate protein sorting by a bilayer-mediated mechanism, in which proteins are targeted to bilayers whose hydrophobic thickness matches the hydrophobic length of their TMD. Sorting in the generally cholesterol-poor Golgi bilayers would involve the lateral partitioning of plasma membrane proteins with longer TMDs into cholesterol-enriched membrane domains (now called lipid rafts; Brown and London, 1998; Simons and Ikonen, 1997), whereas Golgi-resident proteins, with shorter TMDs, would be excluded from the cholesterol-enriched membrane domains (Fig. 1 *A*). The increase in membrane cholesterol content along the secretory pathway further was proposed to reflect a preferential incorporation of cholesterol-enriched domains into forward moving transport vesicles, which therefore would account for protein sorting.

Because of the role of the hydrophobic length of the TMD in sorting, and further because cholesterol depletion leads to mistargeting of plasma membrane proteins, the association of membrane proteins with cholesterol-enriched domains is currently viewed as a potential sorting mechanism (Bagnat et al., 2001; Dumas et al., 1999; Keller and Simons, 1998).

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Abbreviations used: AA, amino acids; d_0, bilayer hydrophobic thickness; f_{Chol}, cholesterol mole fraction; H_b, bilayer spring constant; K_a, area-compression modulus; K_c, bending modulus; l, protein hydrophobic length; r_0, protein radius; TMD, transmembrane domain.

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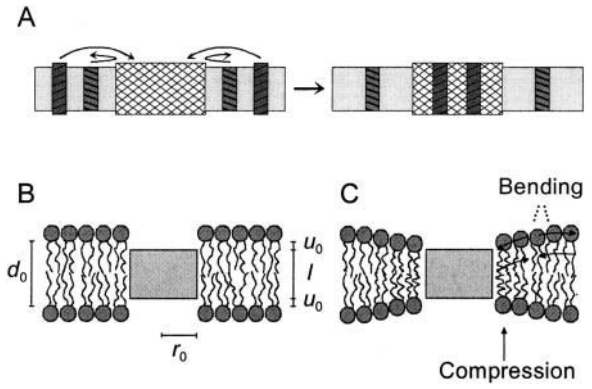

FIGURE 1 (*A*) Lateral sorting of membrane proteins (*dark-hatched*) between thin, cholesterol-poor bilayer domains (*light gray*) and thicker, cholesterol-enriched bilayer domains (*cross-hatched*). The proteins will tend toward the domain in which there is hydrophobic match between the protein length and the bilayer thickness. (*B*) In a nondeformable lipid bilayer, a mismatch between the hydrophobic thickness of the bilayer and the protein hydrophobic length leads to exposure of hydrophobic surface to the aqueous surroundings. (*C*) In a deformable bilayer, the hydrophobic coupling between the protein and the bilayer induces a bilayer deformation.

Although the mechanism(s) underlying the genesis and maintenance of the Golgi complex remain unresolved (cf. Check, 2002), a general feature of all sorting mechanisms is a lateral segregation of proteins between different compartments, which eventually become part of vesicles involved in forward or retrograde transport (cf. Mellman and Warren, 2000). That is, even if proteins destined for the plasma membrane move forward by cisternal maturation (Check, 2002; Munro, 1998), the latter process would involve retrograde transport of Golgi-resident proteins. Also in this case the ability of a membrane protein to associate with cholesterol-enriched domains would serve as a sorting mechanism—provided the retrograde transport vesicles are formed from phospholipid-rich domains of the Golgi bilayers (Munro, 1998).

Bilayer-based sorting, being a physical mechanism, will be operative generally; but it may not be sufficient for effective protein sorting. Targeting to different membrane compartments, for example, can involve sequence-dependent recognition signals (e.g., Bonifacino and Dell'Angelica, 1999), which will exert their action in conjunction with the bilayer-mediated sorting mechanism. The important question thus becomes: how large an impact might a bilayer-based sorting mechanism have on overall protein sorting?

General support for a bilayer-mediated sorting mechanism was obtained in studies on the insertion of hydrophobic α-helices into synthetic lipid bilayers, which correlates with the bilayer thickness and cholesterol content (Ren et al., 1997; Webb et al., 1998). Nevertheless, it is not clear if cholesterol-induced changes in bilayer thickness are sufficient to regulate protein sorting or whether changes in other bilayer properties, such as the material properties, also need to be involved. The adsorption of amphipathic peptides to a lipid bilayer thus varies as a function of the area-compression modulus (cf. Vidal et al., 2002). To address this uncertainty, we have examined the energetic feasibility of a sorting mechanism based on cholesterol-induced changes in the physical properties of lipid bilayers.

We evaluated the feasibility of a simple bilayer-based sorting mechanism by considering the energetic consequences of a mismatch between the hydrophobic thickness (d_0) of a lipid bilayer and the hydrophobic length (l) of the TMD of a membrane protein. If the bilayer were rigid, and its thickness were invariant, a mismatch between d_0 and l would incur an energetic cost that would arise because of the energetic penalty of exposing hydrophobic residues to water (Fig. 1 *B*; also see Tanford, 1980). If the bilayer were just a thin sheet of liquid hydrocarbon, stabilized by the polar headgroups, the hydrophobic coupling between the TMD and the bilayer core would cause the bilayer to adjust locally to the hydrophobic length of the TMD. But lipid bilayers are neither rigid nor thin sheets of liquid hydrocarbon; they are elastic liquid crystals with well-defined material properties (Bloom et al., 1991; Helfrich, 1973; Mouritsen and Andersen, 1998). Consequently, when $d_0 \neq l$, the hydrophobic mismatch will induce an elastic bilayer deformation, in which the acyl chains in the vicinity of the TMD are extended or compressed and also splayed relative to each other, which will incur an energetic cost (Fig. 1 *C*; also see Mouritsen and Bloom, 1984). Because the bilayer deformation energy contributes to the cost of inserting a membrane protein into a lipid bilayer domain, protein sorting will be determined by both the bilayer thickness and material properties (the resistance to compression/extension and bending/splay). As cholesterol alters these bilayer properties, it should, in principle, effect protein distribution—the question becomes whether the cholesterol-induced changes are large enough to be of consequence.

Cholesterol-enriched membrane domains also are enriched in sphingolipids (Simons and Ikonen, 1997.) In the present analysis, however, we consider only the effects of cholesterol because there are insufficient data to quantitatively evaluate the combined effects of sphingolipids and cholesterol. Based on their effects on bilayer thickness (Holthuis et al., 2001) and material moduli (McIntosh et al., 1992), however, the presence of sphingolipids will only further potentiate the effects of cholesterol.

We use the theory of elastic liquid crystal deformations (Huang, 1986) to evaluate the ability of cholesterol to regulate protein sorting by changing the physical properties of lipid bilayers. The results show that cholesterol-induced changes in bilayer thickness and material properties indeed can effect protein sorting. If cholesterol altered only the bilayer thickness, however, the energetic consequences of a hydrophobic mismatch would be rather modest—and the sorting would be less efficient. But the combined effects of the changes in bilayer thickness and material properties are substantial, and the energetic cost of a bilayer deformation is

of sufficient magnitude to regulate sorting, be it alone or in combination with other sorting mechanisms (Gleeson, 1998; Opat et al., 2001). The fact that cholesterol-enriched lipid domains are also enriched in sphingolipids will further increase the effects of cholesterol on protein sorting.

THEORY

Elastic bilayer deformations and the bilayer spring constant

When the hydrophobic interactions between a symmetric bilayer and an embedded inclusion are strong enough to ensure that there is no exposure of hydrophobic residues, the depth of the deformation in each monolayer (u_0), will be $(d_0 - l) / 2$ (Fig. 1 *C*). The associated bilayer deformation energy (ΔG_{def}) will be the sum of contributions from bilayer compression, which varies with u_0 and the area-compression modulus (K_a), and monolayer bending, which varies with the monolayer curvature (c) and the bending modulus (K_c) (Fig. 1 *C*). In addition to these continuum contributions, there will be a contribution from the local lipid packing around the protein, which will tend to increase the deformation energy above the continuum contribution (May, 2000; Nielsen and Andersen, 2000; Nielsen et al., 1998). In the following analysis we define ΔG_{def} as the bilayer deformation energy given by the continuum contributions when the cost of local lipid packing is neglected (see Nielsen and Andersen, 2000, for a detailed discussion of this issue).

The formal expression for the bilayer deformation energy (Dan et al., 1994; Helfrich and Jakobsson, 1990; Huang, 1986; Nielsen and Andersen, 2000; Nielsen et al., 1998) is

$$\Delta G_{def} = \int_{r_0}^{\infty} \left(K_a \cdot \left(\frac{2u}{d_0} \right)^2 + K_c \cdot (c_1 + c_2 - c_0)^2 \right) \cdot \pi \cdot r dr - \int_{r_0}^{\infty} K_c \cdot c_0^2 \cdot \pi \cdot r dr, \quad (1)$$

where r_0 denotes the radius of the inclusion, r is the distance from the center of the inclusion, c_1 and c_2 are the principal curvatures of the monolayer, and c_0 is the equilibrium curvature of an isolated monolayer.

Eq. 1 appears forbidding; but its exact solution is a second order polynomial, which reduces to a particularly simple expression when $c_0 = 0$,

$$\Delta G_{def} = H_B \cdot (2u_0)^2, \quad (2)$$

where the spring constant (H_B) is determined by K_a, K_c, d_0, and r_0. H_B can be determined for any choice of K_a, K_c, r_0 and d_0 using the scaling relations derived by Nielsen and Andersen (2000) (see Appendix). In addition to the bilayer material constants, the value of H_B is determined also by local lipid packing around the protein; and estimates for H_B differ threefold depending on whether this contribution is included or not (Nielsen and Andersen, 2000).

Eq. 2 not only is the analytical solution to Eq. 1, it also describes well the effects of changes in bilayer thickness on the function of gramicidin channels (Lundbæk and Andersen, 1999). Moreover, the spring constant, determined using gramicidin channels, is in good agreement with predictions based on the elastic bilayer model using independently obtained material moduli and including the constraints on lipid packing. In an attempt to ensure that we are not overestimating the consequences of a hydrophobic mismatch, we will in the following assume that there are no constraints on local lipid packing around the protein, however. The present calculations thus should represent lower estimates of the bilayer contributions to protein sorting.

The importance of hydrophobic mismatch is a general feature of analyses of protein-bilayer interactions, and ΔG_{def} calculated using Eq. 2 is in general agreement with results obtained using other methods (Mouritsen and Bloom, 1984; Fattal and Ben-Shaul, 1993; Ben-Shaul et al., 1996; Bransburg-Zabary et al., 2002); but Eq. 2 provides for a particularly convenient method to evaluate the effects of cholesterol on ΔG_{def}, as the value of H_B in the presence of cholesterol can be calculated from experimentally determined values of K_a, K_c, and d_0 (Nielsen et al., 1998; Nielsen and Andersen, 2000).

How to determine H_B (and the effects of cholesterol)

Membrane phospholipids tend to have a saturated acyl chain at the *sn*-1 position and an unsaturated acyl chain at *sn*-2, and 1-stearoyl-2-oleoyl-phoshatidylcholine (SOPC) has been proposed as a prototypical membrane phospholipid (Marsh, 1990; Needham, 1995). Moreover, K_a for an SOPC:Cholesterol (SOPC:Chol) bilayer at an SOPC:Chol molar ratio 1:1, 781 ± 45 pN/nm (mean ± SD) (Needham and Nunn, 1990), is comparable to K_a in red blood cell membranes, 450 pN/nm (Evans and Skalak, 1979) and in plasma membrane blebs from rabbit skeletal muscle, 490 ± 88 pN/nm (mean ± SD) (Nichol and Hutter, 1996). We therefore evaluate the ability of cholesterol to regulate membrane protein sorting, by calculating the effects of cholesterol on the ΔG_{def} associated with accommodating an integral membrane protein in SOPC and SOPC:Chol bilayers.

We first consider the effects of cholesterol on the sorting of an integral membrane protein with a single α-helical TMD of radius, $r_0 = 0.65$ nm (Voegler Smith and Hall, 2001). Such a TMD will remain in an α-helical conformation irrespective of the hydrophobic mismatch with the surrounding bilayer (Zhang et al., 1992). The hydrophobic thickness, d_0, of an SOPC bilayer is ~3.0 nm (Rawicz et al., 2000), and the addition of 50% cholesterol to a phospholipid bilayer increases d_0 ~10% (Nezil and Bloom, 1992). We therefore set d_0 to be 3.0 nm for the SOPC bilayer and 3.3 nm for the SOPC:Chol (1:1) bilayer. We further assume that the bilayer thickness varies as a linear function of the cholesterol mole fraction.

For the present calculations, we use the values for K_a and K_c in SOPC and SOPC:Chol (1:1) bilayers measured by Evans and Rawicz (1990) and Needham and Nunn (1990) (Table 1). These values may be underestimated by up to 20% (cf. Rawicz et al., 2000); but they were obtained using similar criteria (none of our conclusions would be affected if we used the larger values for the moduli). Using the scaling relations in Nielsen and Andersen (2000), we thus find H_B to be 4.1 kcal/(mol nm^2) and 13.1 kcal/(mol nm^2) in SOPC and SOPC:Chol (1:1) bilayers, respectively.

RESULTS

Energetics of a hydrophobic mismatch between a single α-helix and its host bilayer

Using Eq. 2 and the above values for H_B we calculate the ΔG_{def} contribution to the insertion energy for an α-helix in an SOPC or an SOPC:Chol (1:1) bilayer. Fig. 2 shows the results for helices of 15–20 AA, corresponding to l between 2.25 and 3.00 nm. Because the hydrophobic length of a 20 AA α-helix matches the hydrophobic thickness of SOPC, ΔG_{def} is zero in this bilayer; the addition of cholesterol has only a modest effect on ΔG_{def}, which increases to 1 kcal/mol. For the 15 and 17 AA helices, however, cholesterol causes

TABLE 1 Bilayer material moduli

	K_a/(pN/nm)	K_c/(pN nm)
SOPC	193 ± 20*	90 ± 6†
SOPC:Chol (1:1)	781 ± 45*	246 ± 39†

Mean ± SD.
Material moduli measured by *Needham and Nunn (1990); †Evans and Rawicz (1990).

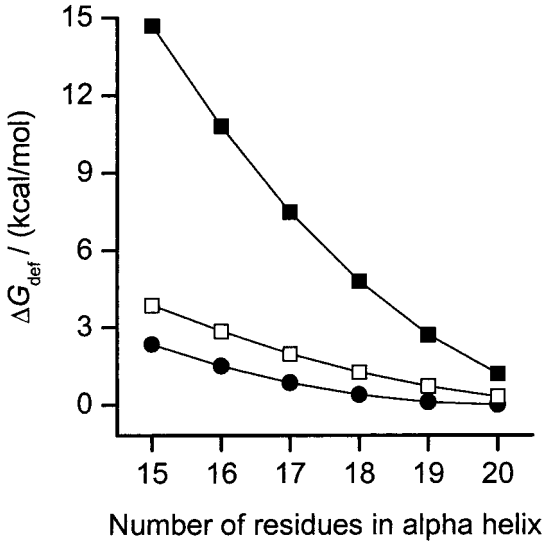

FIGURE 2 ΔG_{def} of inserting α-helices having 15–20 AA into SOPC (●); SOPC:Chol (1:1) bilayers (■); and a bilayer with a thickness corresponding to SOPC:Chol (1:1) but with material properties as SOPC (□).

a large increase in ΔG_{def}, which increases from 2 and 1 kcal/mol in the SOPC bilayer to 14 and 7 kcal/mol in the SOPC:Chol (1:1) bilayer. These energies are large enough to provide a mechanistic basis for membrane protein sorting. They also provide an estimate of the energies needed for sequence-specific sorting mechanisms to override the simple bilayer-based sorting. For comparison, the strength of a hydrogen bond is usually assumed to be ~3 kcal/mol, and the energy released by the hydrolysis of one molecule of ATP to ADP is ~9 kcal/mol (Veech et al., 1979).

Given the above results it becomes useful to evaluate the relative importance of the cholesterol-induced changes in bilayer thickness versus the changes in bilayer material moduli. To do so we calculated ΔG_{def} assuming that cholesterol increased only d_0, but had no effect on the material properties. In this situation ΔG_{def} increases only moderately relative to the values in SOPC (Fig. 2). For helices of 15, 17, and 20 AA the increase is eight-, six-, and fourfold less than the full effects caused by cholesterol.

To evaluate the concentration-dependence of the effects of cholesterol on ΔG_{def} we need to know how changes in the bilayer cholesterol content alter K_a, K_c, and d_0. Fig. 3 *A* shows K_a as a function of the cholesterol mole fraction (f_{Chol}) in an SOPC bilayer (Needham and Nunn, 1990).

For $f_{Chol} < 0.3$, cholesterol has only modest effects on K_a; above this value, K_a rises sharply. Mechanical analysis (Evans and Skalak, 1979) show that K_c, K_a, and d_0 are related by:

$$K_c = K_a d_0^2 / b, \quad (3)$$

where the coefficient b (24) is independent of the acyl chain length in both saturated and monounsaturated phosphatidylcholine bilayers (Rawicz et al., 2000). Needham (1995) similarly found b to be invariant among bilayers of varying composition, including cholesterol-containing bilayers. It thus is possible to estimate the cholesterol-dependent changes in K_c from the measured K_a values (see Fig. 3 *A*). From the changes in the material moduli, the cholesterol-dependent changes in H_B and ΔG_{def} (Fig. 3 *B*) can be calculated. Because K_c for SOPC:Chol (1:1), calculated using Eq. 3, differs slightly from the measured value in Table 1, ΔG_{def} will also differ. This difference never exceeds 10%, however. As for the material moduli, the effects of cholesterol on ΔG_{def} are modest below a f_{Chol} of 0.3; above this threshold ΔG_{def} rises sharply.

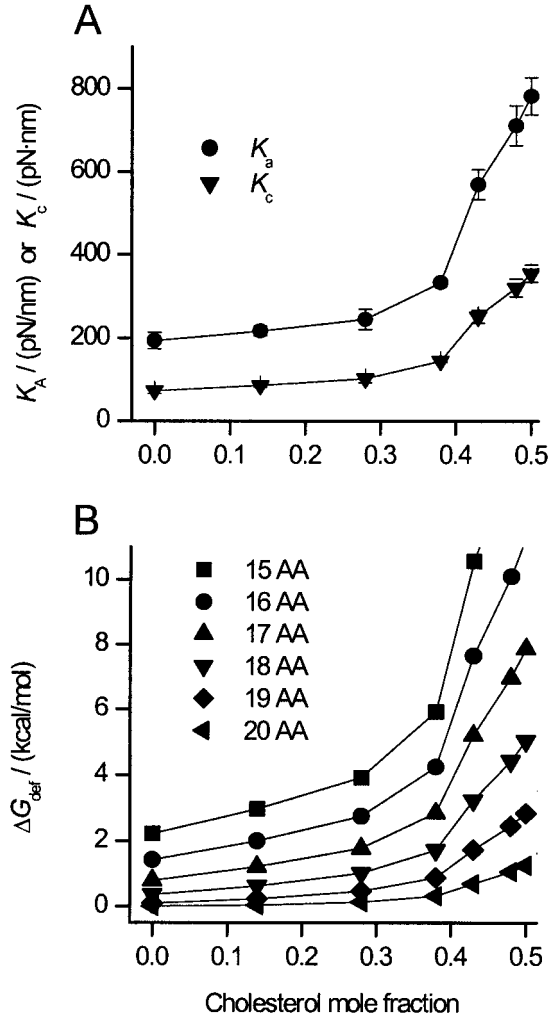

FIGURE 3 (*A*) Effects of cholesterol on the material moduli of SOPC bilayers having various f_{Chol}. (●) K_a measured by Needham and Nunn (1990); (▼) K_c calculated using Eq. 3. (*B*) The effect of cholesterol on ΔG_{def} for α-helices having 15–20 AA.

Cholesterol-induced sorting of single α-helices

The cholesterol-induced changes in ΔG_{def} will affect the sorting of α-helices. Fig. 4 *A* shows the lateral partition

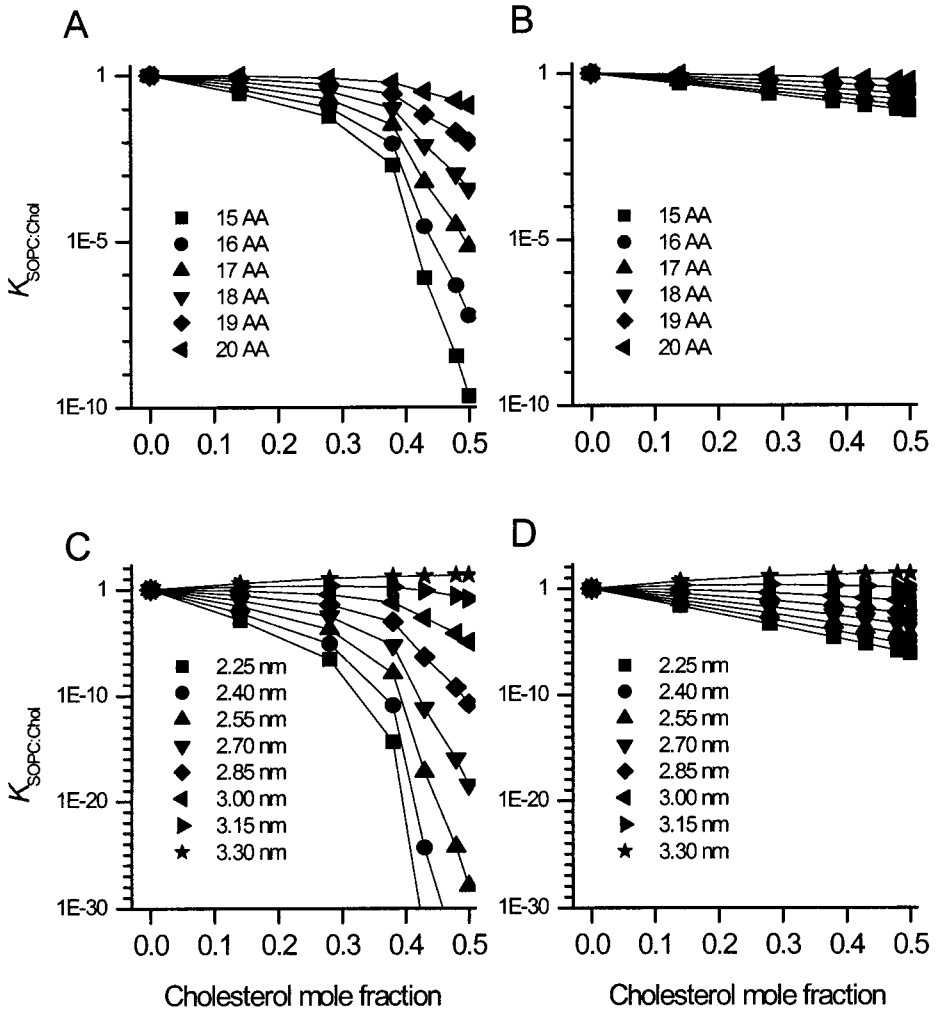

FIGURE 4 (*A, C*) Effects of cholesterol on the lateral partition coefficient, $K_{SOPC:Chol}$, of α-helices (*A*), and multihelical membrane proteins (*C*), of different length, between SOPC and SOPC:Chol bilayer domains. (*B, D*) Effects on the partition coefficient of α-helices (*B*), and multihelical membrane proteins (*D*), of different length, between SOPC and a bilayer domain with a thickness corresponding to SOPC:Chol, but with material properties as SOPC.

coefficient between SOPC:Chol and SOPC bilayer domains ($K_{SOPC:Chol}$) for α-helices of varying length, where

$$K_{SOPC:Chol} = \frac{n_{SOPC:Chol}}{n_{SOPC}} = \exp\left\{-\frac{\Delta G_{def,\,SOPC:Chol} - \Delta G_{def,\,SOPC}}{RT}\right\}, \quad (4)$$

and n_{SOPC} and $n_{SOPC:Chol}$ denote the helix densities, and $\Delta G_{def,SOPC}$ and $\Delta G_{def,SOPC:Chol}$ denote the bilayer deformation energies in the indicated bilayer component. All values of $\Delta G_{SOPC:Chol}$ were calculated using the K_c obtained from Eq. 3.

For $f_{Chol} < 0.3$, cholesterol has only a modest effect on the lateral distribution of single α-helices between cholesterol-free and cholesterol-enriched membrane domains, which means that sorting will be relatively inefficient (Fig. 4 *A*). For $f_{Chol} > 0.4$, cholesterol has a very strong effect. If allowed to distribute freely between SOPC and SOPC:Chol (1:1) bilayer domains, $K_{SOPC:Chol}$ of 20 AA, 19 AA, 18 AA, and 17 AA helices will be 10^{-1}, 10^{-2}, 10^{-4}, and 10^{-5}, respectively. Based on its effects on ΔG_{def} alone, cholesterol thus allows the exclusion of α-helices from a cholesterol-enriched domain. It further allows an accurate discrimination between α-helices that have only modest differences in hydrophobic length.

To evaluate the relative importance of the cholesterol-induced changes in bilayer material properties, we calculated the effects on the sorting of α-helices assuming that cholesterol effected only the bilayer thickness (Fig. 4 *B*). In this situation the effects of cholesterol would be much weaker, and $K_{SOPC:Chol}$ of the 20 AA, 19 AA, 18 AA, and 17 AA helices would be 0.6, 0.35, 0.20, and 0.15, respectively. Thus, if cholesterol altered only the bilayer thickness, $K_{SOPC:Chol}$ for a 20-AA helix would be fourfold that of a 17-AA helix. This is in contrast to the full effects of cholesterol, where $K_{SOPC:Chol}$ of the 20-AA helix is four orders-of-magnitude larger than that for the 17-AA helix (as calculated above). The cholesterol-induced changes in the bilayer material properties thus dramatically potentiate the effects of the changes in bilayer thickness.

Cholesterol-induced sorting of membrane proteins

Because H_B scales with the radius of a bilayer inclusion, ΔG_{def} for multihelical membrane proteins will be larger than for a single α-helix. We show this for a protein with the dimensions of a nicotinic acetylcholine receptor (nAChR). The structure of the nAChR has been determined, and r_0 and l are both ~3 nm (e.g., Unwin, 2000). Using the scaling relations in Nielsen and Andersen (2000), H_B is 21.2 kcal/(mol nm^2) and 68.1 kcal/(mol nm^2) in SOPC and SOPC:Chol (1:1) bilayers, respectively.

ΔG_{def} associated with accommodating a protein with the dimensions of the nAChR in different bilayers was calculated as above. As there is no hydrophobic mismatch in SOPC, ΔG_{def} is zero in this bilayer. In SOPC:Chol (1:1) ΔG_{def} is 6 kcal/mol. If l had been 2.85 nm or 2.7 nm ΔG_{def} would be 1 kcal/mol and 2 kcal/mol in the SOPC bilayer, and 14 kcal/mol and 25 kcal/mol in the SOPC:Chol (1:1) bilayer. In conclusion, for a protein with a radius as the nAChR, ΔG_{def} in a cholesterol-containing bilayer is substantially larger than for an α-helix. Further, a difference in protein hydrophobic length corresponding to only two amino acids in an α-helix (0.3 nm) leads to a difference in ΔG_{def} that is comparable to the energy released by hydrolysis of several ATP molecules.

Fig. 4 *C* shows the effects of cholesterol on the sorting of membrane proteins with radius as the nAChR and with hydrophobic lengths varying between 2.25 to 3.3 nm ($K_{SOPC:Chol}$ was calculated using Eq. 4). Proteins with a hydrophobic length of 3.3 nm are attracted to SOPC:Chol (1:1) because there is no hydrophobic mismatch in this bilayer domain and $K_{SOPC:Chol}$ is 20. In contrast, for shorter proteins with hydrophobic lengths of 3.0, 2.85, and 2.7 nm, the hydrophobic mismatch incurs an energetic penalty and $K_{SOPC:Chol}$ becomes 10^{-5}, 10^{-11}, and 10^{-19}, respectively. In the case of a protein with a hydrophobic length that is in between the thickness of the SOPC and SOPC:Chol (1:1) bilayers, the relation between $K_{SOPC:Chol}$ and f_{Chol} may become biphasic (Fig. 4 *C*, curve for $l = 3.15$ nm). This result arises because, as d_0 increases from the value in SOPC, the protein will tend to reside in the thicker, cholesterol-containing domains as long as $H_B^{Chol} \cdot (d_0^{Chol} - l)^2 < H_B^0 \cdot (d_0^0 - l)^2$, where the H_B and d_0 superscripts denote the values in the absence and presence of cholesterol, respectively. Eventually, however, the increase in H_B^{Chol} and in $(d_0^{Chol} - l)^2$ will cause the inequality to reverse and the protein will tend to reside in the thinner, cholesterol-free domains.

Fig. 4 *D* depicts the effects of an isolated change in bilayer thickness (and constant moduli) on protein sorting. If cholesterol altered only the bilayer thickness $K_{SOPC:Chol}$, for proteins with a hydrophobic length of 3.3 nm and 3 nm, would be 25 and 0.06, respectively. The $K_{SOPC:Chol}$ for the 3.3-nm protein would thus be 400-fold larger than for the 3-nm protein—as opposed to 10^6-fold larger with the full effect of cholesterol. For the lateral distribution between SOPC and SOPC:Chol (1:1) bilayer domains of equal area, this means that if cholesterol affected only the bilayer thickness, the probability of finding the 3.3-nm protein in the SOPC:Chol (1:1) domain would be ~20-fold larger than that of finding the 3-nm protein in this domain. But with the full effects of cholesterol, the probability of finding the 3.3-nm protein in the SOPC:Chol (1:1) domain is ~5 orders-of-magnitude over that of finding the 3-nm protein in this domain.

Is hydrophobic exposure important?

Our results show that a bilayer-mediated sorting mechanism based on bilayer deformation energy is feasible. This raises the question, whether hydrophobic exposure per se (cf. Fig. 1 *B*) ever is important for sorting? For a sufficiently large mismatch between the hydrophobic bilayer thickness and protein length, the incremental change in ΔG_{def} will become so large that it becomes advantageous to expose hydrophobic surface—in the protein or the bilayer—to the aqueous phase, a situation we denote hydrophobic slippage. But the mismatch has to be extreme. When there is hydrophobic slippage, $2u_0$ will differ from $d_0 - l$ (compare with Fig. 1, *B* and *C*), and Eq. 2 will overestimate the energy available for protein sorting.

Following Andersen et al. (1998) and Lundbæk and Andersen (1999), the incremental change in ΔG_{def} is obtained by differentiating Eq. 2 with respect to u_0, and hydrophobic slippage will not occur unless

$$\frac{d\Delta G_{def}(u_0)}{du_0} = 4H_B u_0 > \Delta G^*_{hyd},$$

where ΔG^*_{hyd} denotes the hydrophobic energy associated with exposing a unit length of the hydrophobic bilayer interior. The energetic cost of hydrophobic exposure is ~4.7 kcal/(mol nm^2) (Sharp et al., 1991), such that $\Delta G^*_{hyd} = (2\pi \times r_0) \times 4.7$ kcal/(mol nm^2). For an α-helix with $r_0 = 0.65$ nm, $\Delta G^*_{hyd} = 19$ kcal/(mol nm) In SOPC and SOPC:Chol (1:1) bilayers the magnitude of $4H_B(d_0 - l)$ will be less than this value as long as $|d_0 - l| < 2.4$ nm or 0.7 nm, respectively. For α-helices of 17 AA and longer, hydrophobic slippage will not occur in either bilayer; for 15- and 16-AA helices, slippage will not occur for $f_{Chol} < 0.45$. Similarly for a membrane protein with $r_0 = 3.0$ nm, $4H_B(d_0 - l)$will be less than $\Delta G^*_{hyd} = 89$ kcal/(mol nm) as long as $|d_0 - l| < 1.8$ nm or 0.6 nm in SOPC and SOPC:Chol bilayers (1:1), respectively. This means that for membrane proteins 2.7 nm, or longer, hydrophobic slippage will not occur in either bilayer. We therefore conclude that hydrophobic exposure is unlikely to be important for protein sorting.

DISCUSSION

We have shown that cholesterol-induced changes in lipid bilayer physical properties are more than sufficient to support

a bilayer-mediated protein sorting mechanism based on the lateral distribution of proteins between different bilayer domains. This bilayer-based protein sorting results from changes in the bilayer elastic deformation energy due to a mismatch between the protein length and the bilayer thickness, without invoking hydrophobic exposure. When compared to an isolated (cholesterol-induced) increase in bilayer thickness, the energetic consequences of the associated changes in bilayer material moduli causes a dramatic increase in the sorting efficiency. The bilayer contribution to membrane protein sorting will be operative, and of sufficient magnitude to be important, whether or not the sorting of a given protein also is under the control of other targeting signals.

Cholesterol gradients and implications for bilayer properties and protein sorting

Both cholesterol and proteins are synthesized in the endoplasmic reticulum (ER), and there is an increasing f_{Chol} in the membranes along the secretory pathway (Orci et al., 1981; Wattenberg and Silbert, 1983). Cholesterol thus constitutes ~20% and 50% of the lipids in the Golgi complex and the plasma membranes, respectively (Evans and Hardison, 1985; van Meer, 1989). Further, relatively cholesterol-enriched bilayer domains have been demonstrated in both Golgi (Gkantiragas et al., 2001) and plasma membranes (Pike et al., 2002). The gradual increase in f_{Chol} has been proposed to reflect a selective forward transport of cholesterol (and sphingomyelin)-enriched membrane domains toward the plasma membrane (Bretscher and Munro, 1993); but it could also result from the selective retrograde transport of cholesterol/sphingomyelin-depleted vesicles (cf. Brown and London, 1998; Munro, 1998). In support of such models, the formation of COPI-coated vesicles operating in the early secretory pathway is associated with a segregation of sphingomyelin and cholesterol away from these vesicles; see Brugger et al. (2000). The precise role of these vesicles remain obscure, however; see Mellman and Warren (2000). In either case, a selective enrichment, or depletion, of a membrane protein in the cholesterol-enriched, or cholesterol-depleted, domains would enable protein sorting—as long as the transport vesicles are enriched in only one type of membrane domain. Furthermore, cholesterol depletion will lead to altered protein sorting (cf. Bagnat et al., 2001; Keller and Simons, 1998; Mayor et al., 1998), not only because the domain organization will be disrupted but also because the protein distribution among different domains will become less selective.

The effects of cholesterol on the bilayer material properties are considerable; but cholesterol-enriched lipid domains are enriched also in sphingolipids (Simons and Ikonen, 1997), which will increase both the bilayer thickness (e.g., Holthuis et al., 2001) and material moduli (McIntosh et al., 1992) above the changes induced by cholesterol alone. A bilayer-mediated sorting mechanism based on membrane deformation energy therefore would be even more efficient than indicated by our calculations as previously suggested by Gandhavadi et al. (2002). Specifically, K_a for sphingomyelin:cholesterol (1:1) bilayers is 1799 ± 234 pN/nm (McIntosh et al., 1992)—more than twofold larger than for SOPC:Chol bilayers (Table 1). Assuming that the relation between K_a and K_c in phospholipid:sphingomyelin:cholesterol mixtures is similar to that in phospholipids and phospholipid:cholesterol mixtures, H_B could be twofold larger than the value we use for SOPC:Chol (1:1).

Limitations of the analysis

The present analysis is based on a symmetric bilayer but the phospholipid composition of cellular membranes is asymmetric (e.g., Masserini and Ravasi, 2001; Sprong and van Meer, 2001). It is not known to what extent cholesterol is present in the intracellular leaflet of a cholesterol-enriched lipid raft. In synthetic bilayers, however, domain formation in the two monolayers is coupled (Korlach et al., 1999), which may suggest that the cholesterol content of the two leaflets is similar also in cellular membranes. It is in this context comforting that the deduced energies are large, meaning that even two- to fourfold reductions in the deformation energies would have little impact on our general conclusion that cholesterol-dependent protein sorting, based on hydrophobic matching, is energetically feasible.

Another limitation is that a hydrophobic mismatch between a bilayer and a membrane-spanning protein may alter the lateral distribution of the bilayer lipids around the protein (Andersen et al., 1992; Sperotto and Mouritsen, 1993). In a cholesterol-containing bilayer, where $d_0 > l$, the ensuing bilayer deformation could cause a redistribution of the lipids around the protein such that the local mole fraction of cholesterol would be less than in the bulk, unperturbed bilayer. This would occur because the reduction in bilayer material moduli (and thickness) will reduce the magnitude of ΔG_{def}, as compared to the situation where no redistribution has occurred, which in turn would provide the energetic basis for the redistribution. The quantitative importance of such a lipid redistribution, for the value of ΔG_{def}, is difficult to evaluate; but the presence of cholesterol (2:1) in a dioleoylphosphatidylcholine lipid bilayer causes a twofold increase in H_B, as measured using gramicidin channels (Lundbæk et al., 1996). If the cholesterol-induced increase in the H_B of SOPC bilayers (from SOPC to SOPC:Chol (1:1)) similarly were only a factor 2 (rather than the predicted factor 3), $K_{SOPC:Chol}$ for a 15 AA helix would be 10^{-5}, rather than 10^{-10}, which still would be sufficient for effective sorting.

We conclude that the present analysis constitutes a first-order approximation to the energetics of bilayer-mediated protein sorting, but that the general conclusions are unlikely to be affected by the above limitations.

Cholesterol-dependent protein sorting

Assuming that the mechanical moduli of both leaflets of the bilayer component of a cellular membrane are comparable, the lateral distribution of membrane-spanning proteins between different (cholesterol-poor and cholesterol-enriched) bilayer domains will follow the pattern in Fig. 4. That is, whereas the bilayer-based sorting mechanism is relatively inefficient at $f_{Chol} < 0.3$, the sorting efficiency increases as f_{Chol} is increased above 0.3. Given the change in the slope of the K_a (or K_c) versus f_{Chol} relation, (Fig. 3), there is a threshold in the sorting efficiency, meaning that bilayer-based sorting can occur between bilayer domains that have rather modest differences in their cholesterol concentration —as long as f_{Chol} in at least one of the domains is above 0.3, or so. Moreover, the threshold in the cholesterol-induced sorting would tend to enhance the tendency for the lipid composition of the cholesterol-enriched domains to change as the raft-preferring proteins partition into such domains, or when such domains coalesce into larger structures (compare with Bretscher and Munro, 1993; Dumas et al., 1997; Maer et al., 1999; Sperotto and Mouritsen, 1993). This threshold similarly will serve to strengthen retention mechanisms that rely on vesicle recycling among different compartments (cf. Ghosh et al., 1998).

There is an asymmetry to the cholesterol-induced sorting: the penalty for minor length-thickness mismatches will be significant in the cholesterol-enriched domains, but more modest in the cholesterol-poor domains. This asymmetry is important because it means that bilayer-based protein sorting fundamentally is a proofreading mechanism based on selective exclusion, meaning that proteins with short TMDs will be excluded from cholesterol-enriched bilayer domains —irrespective of the detailed amino acid sequence or structure of the TMD.

Our results provide insight into why the short TMD of Golgi-resident proteins is a conserved feature among eukaryotic cells from mammals to yeast (Holthuis et al., 2001; Levine et al., 2000). The retention of ER resident membrane proteins is likely to be determined, in part, by a similar bilayer-based sorting mechanism: elongating their TMD leads to relocation to the Golgi complex (Pedrazzini et al., 1996; Yang et al., 1997); further elongation causes the proteins to be expressed at the plasma membrane (Yang et al., 1997), and this length-dependent control of protein targeting is observed also with artificial TMDs (Honsho et al., 1998). These observations suggest that a bilayer-based sorting mechanism may be operative generally, between ER and Golgi and between Golgi and the plasma membrane (Yang et al., 1997), and even within the Golgi complex. But in the case of protein sorting between ER and Golgi, bilayer-based sorting is not the sole ER retention mechanism, as there are sequence-specific ER retention/retrieval signals (cf. Yang et al., 1997). Similarly, whereas targeting of the plasma membrane protein, Na^+,K^+-ATPase is controlled, at least in part, by its membrane-spanning domain (Dunbar et al., 2000) in a manner suggesting that a bilayer-based mechanism could be involved, targeting of plasma membrane proteins to apical or baso-lateral membranes generally depends also on sequence-specific signals, e.g., Rodriguez-Boulan and Nelson (1989)—indicating, again, the existence of multiple sorting mechanisms (cf. Mellman and Warren, 2000).

We finally note that bilayer-based sorting arises because biological membranes are not just fluid mosaic structure (Singer and Nicolson, 1972), but elastic bodies with material properties that allow for bilayer deformation, but at a price (cf. Mouritsen and Andersen, 1998). The bilayer elastic properties are such that a hydrophobic mismatch incurs an energetic cost that is sufficient to support bilayer-based protein sorting, without exposure of hydrophobic residues to water. Moreover, given the magnitude of the ΔG_{def} associated with even a modest hydrophobic mismatch, bilayer-based sorting is likely to be a general mechanism, which would be important for the lateral distribution of membrane proteins in any cellular membrane containing cholesterol/sphingolipid-enriched lipid domains. Further, bilayer-based sorting may be important for determining the lateral distribution of proteins whose TMDs vary in length, as seems to be the case for plasma membrane proteins (compare with Bretscher and Munro, 1993, their Fig. 1).

CONCLUSION

Cholesterol-induced changes in bilayer physical properties are sufficient to allow for effective sorting of membrane proteins. The effects of cholesterol are due to the combined impact of changes in bilayer thickness and material properties. The energetic consequences of the changes in the thickness per se, however, are modest; but the associated changes in material properties strongly potentiate the effects of the thickness change. The threshold in the sorting efficiency, induced by the effects on the bilayer material properties, implies that cholesterol-induced protein sorting in effect becomes a proofreading mechanism based on the exclusion of proteins with too short a TMD from the cholesterol-enriched bilayer domains.

APPENDIX

To calculate H_B we make use of the fact that the general solution to Eq. 1 is biquadratic in u_0 and s, the contact slope at the protein-bilayer boundary (Nielsen et al.,1998; Nielsen and Andersen, 2000). For $c_0 = 0$,

$$\Delta G_{def} = a_1 u_0^2 + a_2 u_0 s + a_3 s^2,$$

where a_1, a_2, and a_3 are functions of K_a, K_c, d_0, and r_0, and

$$H_B = \left(a_1 - \frac{a_2^2}{4a_3}\right)/4.$$

If the lipid packing constraints were included, s would be 0 and H_B would be given by a_1. To evaluate the coefficients a_1, a_2, and a_3, we follow Nielsen and Andersen (2000), who calculated reference values, a_1^*, a_2^*, and a_3^* for a set

of reference bilayer-inclusion parameters K_a^*, K_c^*, d_0^*, and r_0^*, and then derived scaling relations that could be used to calculate a_1, a_2, and a_3 (and thus H_B). For any bilayer-inclusion system, the scaling relations have the form

$$a_i(M) = \bar{a}_i \cdot \left(\frac{M}{M^*}\right)^{n_{M,i}} + \hat{a}_i,$$

where a_i is the resulting value of the coefficient in question ($i = 1, 2, 3$), M denotes the material property that is varying, $n_{M,i}$ is the relevant scaling exponent, and $a_i(M^*) = a_i^* = \bar{a}_i + \hat{a}_i$. The values for n_i, $\bar{a}_i$, $\hat{a}_i$ are tabulated in Nielsen and Andersen (2000, their Table 5).

For any given combination of K_a, K_c, r_0, and d_0, we then have that

$$a_i = a_i^* \cdot \left[\frac{a_i(K_a)}{a_i^*(K_a^*)} \cdot \frac{a_i(K_c)}{a_i^*(K_c^*)} \cdot \frac{a_i(d_0)}{a_i^*(d_0^*)} \cdot \frac{a_i(r_0)}{a_i^*(r_0^*)}\right],$$

which allows for the determination of the a_i coefficients and H_B. (When H_b is calculated directly from Eq. 1—Nielsen and Andersen, 2000—we obtain values that are within 10% of the values derived using the scaling relations.)

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