Introduction
Although there is considerable evidence that sphingomyelin is not only concentrated in the plasma membranes of eukaryotic cells but also oriented almost exclusively on the external surface [1–3], there has been some controversy about the actual site of synthesis of plasma membrane sphingomyelin. Originally it was thought to be made in situ in the plasma membrane, which represents the largest pool of sphingomyelin within most cells [4–6]. Subsequently, evidence has been produced for synthesis of sphingomyelin in an internal pool (probably the early Golgi) and for its translocation to the cell surface by a vesicular route similar to that taken by membrane proteins [7–11]. This evidence is based partly on the observation that inhibitors of vesicular transport block the appearance of sphingomyelin at the cell surface and partly on the results of liver cell fractionation, which suggest that sphingomyelin synthetic activity is concentrated in the early Golgi [8, 12, 13].

The work described here demonstrates that although there is a site of sphingomyelin synthesis which is probably in the early Golgi, this is not the site which is responsible for the synthesis of plasma membrane sphingomyelin. The major site of plasma membrane sphingomyelin synthesis appears to be in the endosomal pathway involved in the recycling of plasma membrane.

Materials and methods
Baby hamster kidney (BHK) 21 cells were cultured in 3.5 cm dishes in Glasgow minimal essential medium (MEM) supplemented with 5% fetal calf serum, as described previously [14]. Cells were incubated in the presence of 20 μCi [3H]acetate (New England Nuclear) with or without addition of 0.3 μM monensin (Sigma Chemical Co., Poole, Dorset, U.K.). After 4 h, some samples were incubated for 20 min with Bacillus cereus sphingomyelinase (0.1 unit) to determine the amount of radioactive sphingomyelin which had reached the surface. All the samples were then extracted with 1.9 ml 2:1 v/v methanol/chloroform. Lipids were separated and their radioactivity measured as described previously [15].

Cells were also labelled to equilibrium with [3H]acetate as described previously [15] and were treated with sphingomyelinase to degrade surface sphingomyelin [14]. The resynthesis of sphingomyelin after removal of the sphingomyelinase was measured in control cells and in cells incubated with 10 μM monensin or 5 μg/ml brefeldin A (BFA) over a 3 h period. At the end of this incubation the cells were treated for a second time with sphingomyelinase to determine the amount of sphingomyelin that had been returned to the surface. The effects on sphingomyelin resynthesis of various possible inhibitors of endocytosis were also tested, including N-ethylmaleimide, KCN + deoxyglucose and Al F₄⁻. Mitotic cells were also prepared [16] and their ability to resynthesize sphingomyelin was measured (K.-J. Kallen, P. Quinn and D. Allan, unpublished results).

Effects of monensin and BFA on de novo synthesis of sphingomyelin
The inhibitors of vesicular transport, monensin and BFA, both have potent effects on de novo synthesis of sphingomyelin and its delivery to the surface of BHK cells. BFA prevents delivery of new sphingomyelin to the cell surface (as judged by the amount which is broken down by external sphingomyelinase) and it produces a large increase in labelling of internal sphingomyelin and glucosylceramide [15]. This has been explained in terms of the fusion of endoplasmic reticulum and Golgi membranes induced by BFA [18], which has the effect of diverting ceramide normally destined to be converted to plasma membrane sphingomyelin into early compartments which possess enzymes for the synthesis of glycolipids and internal sphingomyelin [15].

It has been demonstrated previously that monensin prevents the delivery of sphingomyelin to the cell surface [19], but our results show for the first time that there is a decrease in the net synthesis of surface sphingomyelin [20] (Figure 1). A combination of monensin and external sphingomyelinase gives a decrease in sphingomyelin radioactivity which is no greater than their separate effects, showing that monensin is affecting mainly the sur-
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Figure 1
Effects of sphingomyelinase and/or monensin on \[^{3}H\]acetate labelling of sphingomyelin, ceramide and glucosylceramide in BHK cells

Cells were labelled for 4 h as described under Materials and methods, in the presence or absence of monensin (MON). For the last 20 min of the incubation, some dishes were treated with sphingomyelinase (SMase) (0.1 units) before extraction and analysis of lipids. Results are expressed as the percentage of total lipid radioactivity present in sphingomyelin (SM) (solid bars), ceramide (plain bars) and glucosylceramide (hatched bars). Values represent the means of four separate experiments, in each of which triplicate determinations were carried out.

face pool which is susceptible to sphingomyelinase. It is interesting to note that monensin [20], BFA [15, 21] and treatment with exogenous sphingomyelinase [20, 22, 23], which all cause a decrease in plasma membrane sphingomyelin, also promote the conversion of cholesterol to cholesterol ester. This can be seen as a generalized response to a decreased ratio of sphingomyelin to cholesterol in the cell surface and emphasizes the importance which is attached to the maintenance of a normal sphingomyelin:cholesterol ratio in the plasma membrane.

Inhibition of sphingomyelin synthesis by monensin is associated with an accumulation of ceramide (together with some glucosylceramide) which exactly matches the decrease in synthesis of surface sphingomyelin (Figure 1) [20]. Since monensin is thought to block vesicular transport between the medial- and trans-Golgi [24], this suggests that it is interfering with the vesicular transport of ceramide from the medial-Golgi to a more distal site where it can be converted into sphingomyelin. The accumulation of ceramide seems likely therefore to be in the medial-Golgi, where some of it is apparently available to be glucosylated to form glucosylceramide. As noted previously [25], the addition of carbohydrate residues to glucosylceramide to form complex gangliosides is also blocked by monensin and BFA, probably because this processing of glycolipids occurs at sites distal to the medial-Golgi.

Synthesis of internal sphingomyelin in the early Golgi
Despite the above evidence for a site of plasma membrane sphingomyelin biosynthesis distal to the medial Golgi, our work indicates that some de novo synthesis of sphingomyelin does occur in the early Golgi, since about 50% of total new synthesis of sphingomyelin is unaffected by monensin (Figure 1). This is an internal pool which does not reach the cell surface, where it can be degraded by external sphingomyelinase, and could correspond to the internal pool of sphingomyelin (accounting for about 30% of the total by mass) which we have identified previously in BHK cells [26]. The precise location of this pool is unknown, but, based on compositional data [27] and stereological information about the sizes of membrane compartments in BHK cells [28], it appears that the only intracellular
organelles that could accommodate this amount of sphingomyelin are endosomes or endoplasmic reticulum. The internal sphingomyelin seems unlikely to be in endosomes because it labels more rapidly with radioactive choline than does plasma membrane sphingomyelin [26], whereas endosomes would be expected to label more slowly than plasma membrane. One possibility is that synthesis of the internal pool of sphingomyelin is associated with retrograde transport of vesicles from the Golgi to the endoplasmic reticulum, which is known to occur from experiments on protein translocation. This would fit in with the observation that BFA, which is thought to promote this retrograde transport [29], greatly increases the size of the internal pool of sphingomyelin in BHK cells [15].

Resynthesis of sphingomyelin and its return to the cell surface after its degradation to ceramide by exogenous sphingomyelinase

If, as suggested above, surface sphingomyelin is synthesized at a site distal to the medial Golgi, what is the precise location of this site in the cell? An indication that it could be endosomal has been obtained from experiments in which BHK cells have been treated with sphingomyelinase and allowed to resynthesize sphingomyelin after removal of the enzyme [14]. Complete synthesis and return of sphingomyelin to the surface occurs in about 3 h (Figure 2), demonstrating a capacity for sphingomyelin synthesis which is at least five-fold larger than the normal synthesis of this lipid in growing cells. This resynthetic process not only has similar kinetics to plasma membrane endocytic recycling, but also is inhibited by conditions which block endocytic events (K.-J. Kallen, P. Quinn and D. Allan, unpublished results). Thus energy depletion, N-ethylmaleimide, digitonin and AlF$_4^-$, which inhibit endocytic events also block resynthesis of sphingomyelin (Figure 3). In addition, mitotic cells, which have a reduced ability to endocytose, show little capacity to resynthesize cell-surface sphingomyelin (K.-J. Kallen, P. Quinn and D. Allan, unpublished results).

In contrast to their effects on the de novo synthesis of plasma membrane sphingomyelin, neither

**Figure 2**

Resynthesis and return to the surface of sphingomyelin initially degraded by external sphingomyelinase: lack of effect of BFA and monensin

Cells were labelled to equilibrium with [$^3$H]acetate and allowed to resynthesize sphingomyelin after an initial treatment with sphingomyelinase (dashed line). Cells were exposed for a second time to sphingomyelinase after 3 h (dashed lines), when resynthesis was complete. For half of the samples, BFA (5 µg/ml) was included in the medium (filled symbols); sphingomyelin (O); ceramide (V). Lipids were extracted and analysed as described in the legend to Figure 1. Values represent the means ± S.D. of triplicate determinations in a single experiment which was repeated three times with almost identical results. Similar results were obtained in the presence of monensin instead of BFA. Abbreviations are as in Figure 1.
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Figure 3
Resynthesis of sphingomyelin initially degraded to ceramide at the cell surface: effects of various inhibitors

Labeled cells were treated with sphingomyelinase and then allowed to resynthesize sphingomyelin after removal of the enzyme, as described under Materials and methods, either untreated or in the presence of N-ethylmaleimide (0.1 mM) (NEM), digitonin (50 μM) (DIG), deoxyglucose (50 mM) + KCN (100 μM) (-ATP) or AlCl₃ (625 μM) + NaF (30 mM) (AIF₃). Values are expressed as the percentage of the control resynthesis of sphingomyelin and represent the means ± S.D. of three experiments, each of which was based on triplicate individual determinations. Abbreviations are as in Figure 1.

![Graph showing resynthesis of SM relative to control cells](image)

BFA nor monensin have any effect on the resynthesis or return to the surface of sphingomyelin which has been degraded to ceramide on the cell surface (Figure 2). Since both monensin and BFA block vesicular transport through the Golgi cisternae, this important result suggests that ceramide generated in the cell surface does not need to pass through the Golgi apparatus in order to be converted into sphingomyelin. This conclusion is supported by the observation that very little of the ceramide is converted into glycolipids (K.-J. Kallen, P. Quinn and D. Allan, unpublished results), the initial steps of whose synthesis occurs in the early Golgi [13, 30], indicating that ceramide at the cell surface cannot reach the early Golgi either by vesicular or non-vesicular routes. Thus it appears that the resynthesis of sphingomyelin and its return to the surface depends on a non-Golgi site of synthesis, which could be part of the endocytic plasma membrane recycling pathway.

An endosomal site for de novo synthesis of plasma membrane sphingomyelin?
Considering its large synthetic capacity, it is tempting to suggest that the site of sphingomyelin synthesis in the recycling pathway may also be responsible for normal biosynthesis of plasma membrane sphingomyelin, using ceramide which has made its way through the vesicular transport pathway in the Golgi apparatus. If synthesis occurs on the endocytic pathway, then there could be a direct route by which ceramide exiting from the Golgi could reach the endosomal pathway. However the bulk of exocytic membrane flow is directly to the plasma membrane, so most of the entrained ceramide is likely to reach the cell surface. The implication is that most ceramide reaches the endosomal site of sphingomyelin synthesis only after it has passed through the exocytic pathway to the plasma membrane and has undergone endocytosis. Such a tortuous route for transport of ceramide could be consistent with the relatively slow kinetics of plasma membrane sphingomyelin synthesis [26].

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17. Reference deleted
Properties and intracellular localization of phosphatidylinositol transfer protein in Swiss mouse 3T3 cells

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In most mammalian cells the endoplasmic reticulum has been identified as the main site of phosphatidylinositol (PI) biosynthesis [1, 2]. Since the receptor-mediated breakdown of phosphatidylinositol 4,5-bisphosphate (PIP2) and the conversion of PI into PIP, occurs in the plasma membrane [3, 4], the question by what mechanism the level of PI in the plasma membrane is maintained occurs. In a mechanism similar to that proposed for transport of cholesterol [5, 6], it is conceivable that phosphoinositides including PI are transported from the endoplasmic reticulum to the plasma membrane by intracellular vesicle flow [7]. In another proposed mechanism, transfer of PI to the plasma membrane occurs directly by a monomolecular insertion reaction involving the phosphatidylinositol transfer protein (PI-TP) [2, 8, 9].

Recently the interest in the physiological role of PI-TP has strongly increased with the observation that PI-TP in yeast is associated with Golgi structures [13]. Furthermore deletion of the gene encoding PI-TP was shown to be lethal for the organism [14]. The requirement for PI-TP can be bypassed by a mutation in the CDP-choline pathway for phosphatidylcholine (PC) biosynthesis [13, 15]. Since PI-TP has the ability to transfer both PI and, to a lesser extent, PC [9] this observation has been interpreted to indicate that PI-TP has the capacity to control the relative PI/PC composition of the yeast Golgi membranes, which is proposed to be critical to the secretory competence of these membranes.

Properties

The first PI-TP to be purified was that from bovine brain [16, 17]. This protein has a molecular mass of 33 kDa and consists of two isoforms with isoelectric points of 5.3 and 5.6, closely resembling rat and human PI-TPs [18, 19]. This resemblance is reflected in the crossreactivity of the anti-rat PI-TP antibody with PI-TP in yeast and human brain membranes [19]. In fact PI-TP appears to be strongly conserved, since anti-bovine PI-TP antibody reacted with a 35–36 kDa protein in the membrane-free cytosol from mammals, birds, reptiles, amphibians and insects [20]. The sequence of rat PI-TP as deduced from cDNA analysis is composed of 271 amino acid residues and the N-terminal peptide

Abbreviations used: PC, phosphatidylcholine; PI, phosphatidylinositol; PIP2, phosphatidylinositol 4,5-bisphosphate; PI-TP, phosphatidylinositol-transfer protein; PMA, phorbol 12-myristate 13-acetate.

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