Isolation and characterization of filipin-resistant LM cell variants not auxotrophic for sterol

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Abstract A series of LM cell variants resistant to filipin, but not auxotrophic for sterol, was isolated by plating mutagenized, filipin-treated cells on soft agar medium containing no sterol. Cloned variants were assayed for growth in the presence and absence of sterol or unsaturated fatty acid. Filipin-resistant clones whose growth rate was unaffected by the addition of sterol to the medium were further analyzed. Variants were cultured in minimal medium, and plasma membranes prepared from these cultures were analyzed for sterol content, phospholipid head group composition, and unsaturated fatty acyl content. All variants examined showed a decrease in membrane sterol in conjunction with an increase in unsaturated fatty acyl chains in the membrane phospholipids. In addition, several variants exhibited alterations in phospholipid head group composition, including changes in the phosphatidylcholine/phosphatidylethanolamine ratio, or a decrease in sphingomyelin content. These observations imply that several different metabolic lesions can give rise to decreased plasma membrane sterol content (and hence to filipin resistance). The range of phospholipid alterations observed in these sterol prototrophs emphasizes the complex interrelationship between membrane sterol and phospholipid structure.—**Rintoul, D. A., N. Neungton, and D. F. Silbert.** Isolation and characterization of filipin-resistant LM cell variants not auxotrophic for sterol. *J. Lipid Res.* 1982. **23:** 405–409.

Supplementary key words phospholipid metabolism • plasma membranes • unsaturated fatty acid metabolism

It has long been recognized that eukaryotic cellular membranes are composed of many different species of lipid (1), and that particular types of membranes from the same cell often vary widely in lipid composition (1, 2). This heterogeneity extends both to lipid classes, e.g., sterols and various types of phospholipids (2); and even to the level of molecular species, e.g., disaturated vs. diunsaturated species of glycerophospholipids (3–5). The function of this structural variability is still relatively mysterious, although some progress has been made in recent years. One approach to the analysis of this heterogeneity has been to isolate variants that are defective in synthesis of certain membrane lipids, and ascertain the effects of this loss on the other membrane lipids. A particularly fruitful approach in this regard has been the analysis of LM cell variants which are auxotrophic for sterol, first reported by Saito, Chou, and Silbert (6). The selection technique used to obtain these variants relied on the cytotoxic effect of low concentrations of filipin, a polyene antibiotic which binds to membrane sterol and presumably damages the membrane (7). Various clones of LM cells resistant to this treatment have been characterized; most of them are true sterol auxotrophs and do not proliferate in the absence of exogenous sterol. These mutants have been very useful in analysis of the role of sterol in plasma membrane structure and function (5, 8, 9). However, this selection scheme also gave rise to a number of variant clones which did not require exogenous sterol, but which were filipin-resistant nonetheless. One of these variants, known as S1, has been previously characterized (5, 6); it seems to be a leaky auxotroph that can synthesize sufficient sterol to grow, albeit slowly, in the absence of exogenous sterol. In the present paper, we analyze several additional filipin-resistant, sterol prototrophic variants in an attempt to gain some further insight into the role of sterol in eukaryotic plasma membrane structure.

MATERIALS AND METHODS

Cell culture

Stock cultures of cells were grown in Minimal Eagle's Medium (Flow) containing nonessential amino acids and 5% fetal calf or calf serum, in 25-cm^2 plastic flasks (Falcon), at 37°C in a CO_2 incubator. Cloning of cells

Abbreviations: DLP, delipidated fetal calf serum; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; SM, sphingomyelin; TLC, thin-layer chromatography.

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TABLE 1. Generation times of LM cell variants

Medium	S1	Wild Type	N194	R7
		hours		
MEM + 3% DLP	24	20	20	35
MEM + 3% DLP + 20 μg/ml Cholesterol	18	18	20	41
MEM + 3% DLP + 10 μg/ml Oleate	52	18	21	N.D.
MEM + 5% Calf Serum	18	17	22	34

Cells were grown in 2.0 ml of medium in 24-well Linbro dishes, as described in Materials and Methods. Protein concentrations in quadruplicate wells were determined daily for 4 days. Generation times (in hours) were calculated from the slope of a least squares fit to the data points from days 2–4. N.D. = not determined.

was performed as previously described (6) on soft agar plates containing 0.5% w/v bovine serum albumin (Sigma). For plasma membrane preparations, trypsinized monolayer cultures were used to inoculate bottles of Higuchi medium, a minimal medium containing no serum and no sterol source. Cells were grown on rotary shaker baths at 37°C as previously described, and harvested by centrifugation (9).

Membrane preparation and analysis

Plasma membranes were prepared as previously described (9) with the exception that N_2 cavitation after 20 min at 720 psi was used to disrupt the cells, rather than the osmotic lysis procedure described in reference 9. These membranes were assayed for protein according to the method of Peterson (10), and for Na^+, K^+-ATPase, and succinate and NADPH-dependent cytochrome c reductase as previously described (9). Typical preparations were enriched 4 to 7-fold for Na^+, K^+-ATPase as compared to the lysate. Membranes were extracted by the method of Bligh and Dyer (11); lipid classes were separated on silicic acid columns, and analyzed for sterol by gas–liquid chromatography (9). Phospholipid phosphate was assayed, after ashing in $Mg(NO_3)_2$ (Sigma), by the method of Muszbek, et al. (12). Two-dimensional TLC of membrane phospholipids on Silica Gel G was performed as described by Freter, Ladenson, and Silbert (5). Quantitation of phospholipid from TLC plates was achieved by first visualizing the lipid spots with H_2O, scraping the gel, extracting it twice in $CHCl_3$–MeOH–H_2O 5:5:1, and assaying the extract for lipid phosphorus as described above. Recovery of standard lipids from Silica Gel G by this procedure was always 95–100%. Determination of phospholipid acyl groups was performed by transesterifying anhydrous lipids in methanolic-HCl (Supelco), extraction with pentane, and analysis of this extract by gas–liquid chromatography on a 6-ft diethylene glycol succinate column at 160°C. Quantitation was achieved using a Varian CDS 111 integrator.

Growth studies

Serum was delipidated by solvent extraction, as described by Rothblat et al. (13). Cells, 1–2 × 10^4, were inoculated into 2 ml of medium containing appropriate additions, in 24-well Linbro dishes; they were grown at 37°C for various lengths of time, washed twice with phosphate-buffered saline, and assayed for cellular protein by the method of Peterson (10), essentially as described by Rintoul, Sklar, and Simoni (14). Generation times were calculated from a least squares curve-fitting procedure, using data points from the final 3 days of a typical 4-day experiment.

Reagents

Chloroform, methanol, and pentane were from Mallinckrodt. All other chemicals were from Sigma.

RESULTS AND DISCUSSION

Nutritional requirements

Mutagenized cultures were selected in filipin-containing medium, as described by Saito et al. (6). The selection procedure was performed twice. These cultures were plated onto soft agar plates containing minimal medium and allowed to proliferate for 10–12 days, at which time 10 colonies were picked. These clones were labeled R1-R10. An additional variant, N194, was isolated during

TABLE 2. Plasma membrane preparation and characterization—wild type and variant LM cells

Na^+, K^+, ATPase	WT	S1	N194	R1	R2	R4	R5	R7	R9
Purification compared to particulate lysate	4.0	5.7	4.3	2.3	4.5	6.5	3.3	2.2	4.6
μmol PL/mg protein	0.93	0.96	1.04	0.73	1.04	0.84	0.88	0.86	0.86

Cells were grown in suspension in Higuchi Medium + 0.5% bovine serum albumin. Plasma membranes were prepared, assayed for protein, Na^+, K^+, ATPase, and phospholipid as described previously (9). The data shown are averages of at least two separate membrane preparations.

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TABLE 3. Plasma membrane lipid analysis

	WT	S1	N194	R1	R2	R4	R5	R7	R9
Sterol/Phospholipid (mol/mol)	0.45	0.32	0.30	0.28	0.32	0.28	0.30	0.35	0.34
% PE	31	29	31	32	33	35	32	39	33
% PC	40	45	43	44	33	36	43	42	44
% SM	15	14	12	8	14	12	9	5	11
% Other PL	14	12	14	15	19	17	16	14	12
% Saturated fatty in total PL	49	28	32	39	43	38	37	34	33

Cells were grown in suspension in Higuchi medium + 0.5% bovine serum albumin. Plasma membranes were isolated, assayed, extracted, and analyzed as previously described (9). Percentages of PE, PC, SM, and other PL are calculated as percentages of total phospholipid in the extract. These data are averages of at least two separate experiments, in which the individual determinations differed by a maximum of 3%.

a mutant search conducted in 1977. All of these variants were resistant to filipin, and yet could grow in the absence of sterol. Nutritional requirements of these clones were established by analysis of cell growth in the presence and absence of exogenous sterol and/or fatty acid. Representative data are shown in **Table 1.** Clones R3, R6, R8, and R10 were found to be leaky sterol auxotrophs by this method, i.e., cell growth was markedly enhanced by the addition of sterol or 5% calf serum (data not shown). These clones are nutritionally analogous to the variant known as S1 (see Table 1) and were not further characterized. The remainder of the R-series variants, along with N194, were shown to be unaffected by the addition of sterol to the growth medium. In fact, addition of 5% calf serum to the medium, which typically decreases the generation time of the leaky auxotroph S1, had little or no effect on the generation time of these variants. Addition of 10 μg/ml oleate, which had no effect on the growth of the wild type cells, drastically increased the generation time of the S1 variant, and had no effect on the growth of the N194 variant. Such observations imply that these filipin-resistant cells were not starved for either sterol or fatty acid, but rather could synthesize adequate amounts to maintain cell growth in the absence of exogenous lipid. These variants were different from the leaky auxotroph S1 in two ways; they were insensitive to 10 μg/ml oleate, and insensitive to 20 μg/ml cholesterol.

Plasma membrane isolation and characterization

Plasma membranes were prepared from cells grown in minimal medium, and were assayed for marker enzymes, phospholipid content, and protein content as previously described (9). These data are presented in **Table 2.** The plasma membrane preparations from the variant cells were enriched for the surface membrane marker enzyme Na^+, K^+-ATPase, and were seemingly normal with regard to their phospholipid and protein content. More detailed lipid analyses were performed on these enriched plasma membrane preparations. These data are presented in **Table 3.** It can be seen that all the filipin-resistant cells, including the leaky auxotroph S1, were characterized by a plasma membrane sterol/phospholipid ratio that was, on the average, 30% lower than the wild type ratio. This lowered sterol content in the plasma membrane probably accounts for the filipin resistance of these clones. An additional feature of interest was that all the filipin-resistant variants had a lower percentage of saturated fatty acid in the plasma membrane phospholipids. This is consistent with our hypothesis (9, 15) that a major role of sterol in the LM cell plasma membrane is to disrupt the phospholipids and prevent lateral phase separations at the physiological temperature. In the LM cell, lowered plasma membrane sterol is always associated with a lower percentage of saturated fatty acyl chains in the phospholipids, unless oxygenated sterols are used to artificially lower the sterol concentration (16).

Previous observations had led to the conclusion that an additional metabolic response to sterol depletion in the LM cell was an increase in the PC/PE ratio, which should also serve to disrupt or "fluidize" the phospholipid bilayer (5). However, it can be seen from Table 3 that this was not always the case. Three of the variants did indeed have a higher PC/PE ratio; these were S1, N194, and R9. Three had normal PC/PE ratios; these were R1, R5 and R7. Two variants actually had very low PC/PE ratios, these were R2 and R4. These data imply that an elevated PC/PE ratio is often observed in sterol-depleted cells, but is by no means an obligate response to sterol depletion. The mechanism of coupling of this phospholipid alteration to sterol metabolism remains obscure.

An additional means by which the cell could prevent lateral phase separations in the plasma membrane might be to decrease the concentration of sphingomyelin (SM) in this membrane. Previous work had implicated SM as a component in the gel phase lipid observed upon sterol loss from the membrane (9). It therefore seems theoret-

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ically possible that a reduction in plasma membrane SM could lead to lowered plasma membrane sterol. Such a reduction had not been previously observed, however. The data in Table 3 suggest that such a response exists in the case of the variants R1, R5, and R7, where SM in the membrane was approximately one-third to one-half that found in the wild type cell. Interestingly, these variants all had "normal" PC/PE ratios in the membrane. These data imply that the primary defect in these variants could be a decreased rate of sphingomyelin synthesis, or an increased rate of spingomyelin turnover.

These lipid analyses, coupled with the growth studies, offer some insight into the role of sterol in the LM cell plasma membrane. The first conclusion from these data is that many different types of filipin-resistant variants can be isolated; the two common denominators among these variants were a lowered sterol/phospholipid ratio, and a decrease in saturated fatty acyl content in the phospholipids. Some of these variants were auxotrophic for sterol, like the S2 clone previously described (5, 6, 8, 9). Some were leaky auxotrophs, and regained normal growth and lipid composition upon the addition of sufficient sterol to the growth medium. Still others were apparently competent in sterol synthesis, as they grew adequately without sterol and were not affected by the addition of cholesterol to the growth medium. We interpret this to mean that, in these cells, sterol availability was not rate-limiting for growth. Such variants could possibly be partially blocked in sterol biosynthesis, but this seems unlikely when they are compared to the known leaky auxotroph S1. Addition of sterol markedly enhances the growth of S1, but has no effect on the growth of the N194 variant or the series of R variants presented in Table 3. In addition, gas–liquid chromatographic analysis of the sterol synthesized by these variants (data not shown) indicated that no unusual sterols were synthesized, again in contrast to S1, which contains a significant fraction of an unique sterol component (6). This result implies that the lowered level of sterol in the plasma membrane of these cells was not due to a block or partial block in sterol synthesis, but rather to some other, undefined, metabolic lesion.

The second conclusion that can be drawn from these data is that the several lipid compositional alterations correlated with sterol depletion, first observed in the S2 auxotroph, can also be detected in non-auxotrophic filipin-resistant clones. It should be emphasized that these plasma membrane lipid alterations are merely correlated with sterol depletion. Further investigations will be required to demonstrate unequivocally any obligate coupling of sterol depletion with the plasma membrane lipid changes observed in these variants. However, all these variants showed a decrease in saturated fatty acyl chains in the membrane phospholipids. In addition, several variants had an increased PC/PE ratio. A novel correlate of sterol depletion, which was predicted from previous observations (9), was a decrease in plasma membrane SM. All these alterations have the common property of decreasing the probability of lateral phase separation in the membrane. However, two of the variants, R2 and R4, had a *lowered* PC/PE ratio and nearly normal levels of SM in the membrane. It is possible that these two mutants have an increase in very long-chain, polyunsaturated fatty acyl groups, or perhaps an increase in other disruptive phospholipid species such as lysoglycerophospholipid. It is noteworthy that both these mutants had a slightly elevated percentage of "other" phospholipids in the membrane. The nature of this alteration is not understood at present and will require further investigation. In addition, it should be emphasized that these several phospholipid alterations did not necessarily all occur in each variant. Coupling of sterol depletion with decreased saturation of the phospholipids seems to be fairly tight, since all the variants examined so far exhibited this correlation. However, alterations in phospholipid head group composition were not necessary correlates of sterol depletion. The increase in the PC/PE ratio was sometimes seen; at other times a decrease on the SM content was observed. It is interesting to note, however, that in no case were both these types of phospholipid alterations detected in the same variant. Finally, the existence of variants where decreased sterol is associated with both a lowered PC/PE ratio and a normal amount of SM also implies the existence of hitherto unsuspected mechanisms for modulation of plasma membrane lipid structure.

In conclusion, the above observations generally reinforce our previous hypothesis that a major role of sterol in LM cell plasma membranes is to prevent the formation of gel phase domains at the physiological temperature. Lowered plasma membrane sterol content is always associated with an increase in unsaturated fatty acid content of membranes, and often associated with phospholipid head group alterations of a nature that should decrease the probability of such gel phase formation (2). The growth inhibition by unsaturated fatty acid, observed in the case of the leaky sterol auxotroph S1 (Table 1), is also intriguing. It is possible that this observation provides evidence for a "condensing" function of sterol in the LM plasma membrane under conditions where the phospholipids are very unsaturated. In this case further incorporation of unsaturated acyl groups, in conjunction with limited sterol, might lead to increased passive membrane permeability.[3] This increased permeability might be the basis for the observed decrease in growth rate. However, other unexplained alterations in plasma

[3] Baldassare, J. J. Unpublished observations.

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membrane lipid composition were also noted, and their existence implies regulatory mechanisms that are not clearly defined at present. The interrelationship between plasma membrane phospholipids and sterol is obviously very complex, and mechanisms that regulate sterol and phospholipid metabolism may be coupled at several points (16–18). Further analysis of the variants described in this report should enable us to gain further insight into the functional consequences of membrane lipid heterogeneity.

The authors would like to thank Shuh-Mei Chou for assistance in the preparation of membranes from LM cells. This research was supported by grants 1 F32 GM06641-01 and GM 16292 from the National Institutes of Health, and grant BC198D from the American Cancer Society.

Manuscript received 24 August 1981 and in revised form 20 November 1981.

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