Cholesterol Availability Modulates Myoblast Fusion

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ABSTRACT   The requirement of cholesterol for myoblast fusion has been linked to the primary step in the fusion process, calcium-dependent aggregation (recognition). Inhibition of cholesterol synthesis with 25-hydroxycholesterol or compactin in the absence of exogenous lipid dramatically inhibits calcium-mediated aggregation and concomitant fusion within several hours. Restimulating cholesterol synthesis or supplying exogenous cholesterol rapidly restores aggregation activity. Over this time period, however, the sterol:phospholipid ratio is unaltered, suggesting a local rather than a general membrane cholesterol requirement for the expression of aggregation activity. The aggregation response to a change in sterol availability occurs on a shorter time scale than that required to inhibit the synthesis of the protein(s) with aggregation activity; thus, the cholesterol-requiring step is posttranslational. We suggest that the assembly or maintenance of the aggregation activity depends on a continued local supply of cholesterol.

Cholesterol is a ubiquitous constituent in the surface of animal cells. Despite a wealth of physical studies on natural and model membranes demonstrating that cholesterol plays a central role in determining membrane lipid structure, its role in cell physiology remains unclear. Recently, we (9, 14) reported that myoblast fusion into multinucleated myotubes is inhibited when myoblasts are grown in a lipid-depleted medium (LDM)[1] containing 25-hydroxycholesterol (25-OH), a potent inhibitor of cholesterol synthesis at the level of hydroxymethylglutaryl-CoA reductase (2, 11). This inhibition appears manifest at what we have termed the recognition stage of myoblast fusion, the first of a sequence of stages that myoblasts traverse during the fusion process (13, 14). The nature of this connection between cholesterol availability and myoblast recognition is the subject of the present communication.

MATERIALS AND METHODS

Cell Culture

Primary explants of 11-d chick breast muscle were prepared as described previously (13). Lipid-depleted fetal calf serum and LDM containing biotin were also prepared as described previously (9). Dried lipid-depleted serum protein was reconstituted in water at 46 mg of protein per milliliter. Primary myoblasts were seeded in calcium-free LDM at a density of 1.3×10^5 cells/cm^2: 2.5×10^6 cells/60-mm plate (5 ml medium) and 8×10^6 cells/100-mm plate (12 or 15 ml of medium). EGTA (1 µg/ml) was added after 5 h to chelate Ca^{2+} present in the serum. Cytosine arabinoside (1.2 µg/ml) was added 28–30 h after seeding to kill fibroblasts.

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[1] *Abbreviations used in this paper:* LDM, lipid-depleted medium; 25-OH,25-hydroxycholesterol; LDL, low density lipoprotein; GLC, gas-liquid chromatography; BSS, balanced salt solution.

Aggregation and Fusion Assays

Cultures grown in LDM for 52 h were detached from the plates with EDTA. Calcium-mediated aggregation was assayed at 52 h according to the procedure of Knudsen and Horwitz (13). The fraction of cells in aggregates of two or more as judged by phase microscopy was scored after 20-min incubation at 37°C on a rotary shaker. During the course of these experiments it was found that the fraction of cells in Ca^{+2}-dependent aggregates could be doubled (from 20–25% to 45–50%) by substituting Hanks balanced salt solution (BSS) for phosphate-buffered saline (14). In all experiments, the percent of aggregating cells for control cultures varied between 20 and 50%. Because of the variation in absolute values of controls, the data are expressed as percent of control. Background aggregation (calcium-independent) averaged <10% of total cells.

Fusion was scored as the percent of total nuclei in myotubes (cells containing two or more nuclei in a cluster or line within a single cytoplasm) as described (9). Ca^{+2} was added at 52 h to a final concentration of ~1.8 mM.

Measurement of Sterol and Protein Synthesis Rates

To measure lipid synthesis, cultures (60-mm plates) were pulse-labeled for 2 h with 0.66 µCi/ml [^{14}C]acetate and harvested as described in the accompanying paper (5). The radioactivity incorporated in sterol was analyzed after thin-layer chromatography of Bligh-Dyer lipid extracts (3) using the solvent system of Freeman and West (7). To measure the protein synthesis rate by myoblasts in the presence of cycloheximide, [^{3}H]leucine (2 µCi/ml) was added simultaneously with 10 µg/ml cycloheximide. At 10, 30, 45, 70, and 100 min thereafter, the radioactivity incorporated into TCA-precipitable material was determined by adding 5% TCA directly to the culture plates as described in the accompanying paper (5). The rate of glycoprotein synthesis of 52-h myoblast cultures was estimated by adding [^{14}C]fucose (0.5 µCi/ml) after 50 h in culture and measuring the radioactivity incorporated into cold 5% TCA-precipitable material at 52 h. To determine the effect of tunicamycin on glycoprotein synthesis, 2 µg/ml tunicamycin was added 2, 4, 6 and 8 h before incorporation of [^{14}C]sugar into TCA precipitable material was measured at 52 h.

Analysis of Lipid Content of Total Cellular Membranes

The method of Bligh and Dyer (3) was used to extract lipids from cells harvested with trypsin in versine. The CHCl₃ phase was analyzed for sterol content by gas-liquid chromatography (GLC) (9), and phospholipid phosphate by the procedure of Bartlett (1).

Preparation and Lipid Analysis of Plasma Membranes

Plasma membranes were prepared according to the method of Schimmel et al. (16) as modified by Kent et al. (12). Approximately 20 100-mm plates of primary cultures were scraped with a Teflon scraper or rubber policeman for each analysis. The plasma membrane (5% of total protein of homogenate) was identified by a 7.4-fold enrichment of ouabain-sensitive Na⁺,K⁺ ATPase (16) and a 2.5-fold enrichment in the sterol:phospholipid ratio. GLC was used to determine plasma membrane sterol (see above). The phosphate assay was too insensitive for the small amounts of lipid we had to analyze. Instead, phospholid content was determined by transesterifying half of the CHCl₃ layer of the Bligh-Dyer extraction. The mass of total fatty acid was determined by GLC using 19:0 as an internal standard (added before lipid extraction). Inasmuch as diglyceride and triglyceride may account for ~5% of total glycerides in the plasma membrane (12), this method can only estimate the phospholipid (PL) content within ~5%.

Preparation of Liposomes

Liposomes were prepared by sonicating 10 μmol each of egg lecithin (purified by alumina chromatography [17]) and cholesterol in 1 ml of Hanks' BSS for 1 h on ice with a Branson sonicator (Branson Sonic Power Co., Danbury, Conn.) at a power setting of 3. The large multilamellar vesicles and titanium debris were sedimented at 5,000 rpm in a Sorvall rotor for 10 min (DuPoint Instruments-Sorvall, DuPont Co., Newtown, Conn.). The liposomes in the supernate were sterilized with a 0.45 μm Millipore filter (Millipore Corp., Bedford, Mass.), flushed with argon, and used within 2 d.

Preparation of Low Density Lipoproteins (LDL)

LDL, density 1.006-1.063, was isolated by standard flotation procedures (8, 15), dialyzed against three two-liter vol of saline over 2 d, and sterilized by Millipore filtration. Aliquots were taken for protein and sterol content. The sterol was 75% esterified cholesterol, and the protein:cholesterol ratio was 1:1.

Reagents

25-OH and coprostanol were obtained from Steraloids, Inc., Wilton, N. H. [2-¹⁴C]acetate (40-60 mCi/mmol), [1-¹⁴C]fucose (40-55 mCi/mmol), and L-[3,4,5-³H]leucine (>100 Ci/mmol) were purchased from New England Nuclear, Boston, Mass. Biotin, cholesterol, and mevalonolactone were obtained from Sigma Chemical Co., St. Louis, Mo. Biotin was dissolved in 50% ethanol, cholesterol in absolute ethanol, and mevalonolactone in H₂O (pH ≃ 3.5). Tunicamycin was dissolved in ethanol. Compactin was sonicated and hydrolyzed to the acid form in 0.1 N NaOH and diluted in buffer (4).

RESULTS

Chick primary myogenic cells cultured for 2 d in the absence of Ca^{+2} undergo rapid synchronous fusion when Ca^{+2} is reintroduced (18). The optimum time for Ca^{+2} addition is ~52 h after seeding. The fusion process can be described as a sequence of events beginning with myoblast specific recognition, which can be monitored in a suspension assay using cells detached from tissue culture plates with EDTA (13).

Inhibitions of Sterol Synthesis Inhibit Ca^{+2}-dependent Aggregation and Fusion

25-OH and compactin are two inhibitors of cholesterol synthesis that inhibit hydroxymethylglutaryl-CoA reductase by different mechanisms (2, 4, 11). When chick myoblasts are cultured for 2 d in LDM containing 1 μg/ml of either 25-OH or compactin, Ca^{+2}-mediated aggregation is almost completely inhibited (see Table I). The percent of total cells that aggregate is reduced from an average of 38% to <2%. There are parallel inhibitions of fusion, which is scored 20 h after the addition of Ca^{+2} to 52-h, EGTA-blocked cultures. The rate of sterol synthesis after 2 day's incubation with compactin or 25-OH declines 70 and 89%, respectively. The sterol:phospholipid ratio of total cell lipids declines 25%, and this ratio in the plasma membrane declines 40%. Chick myoblasts cultured in LDM contain only nonesterified sterol, which consists of cholesterol and desmosterol in a ratio of 9:1. When cholesterol synthesis is inhibited, the amount of desmosterol drops to 2% of total sterol.

We sought to determine whether the inhibition of aggregation correlates more closely with the change in the rate of sterol synthesis or in the general level of membrane sterol. To do this, we compared the time-course of the 25-OH-induced inhibition of aggregation with that of sterol synthesis using varying inhibitor concentrations. The rate of inhibition of sterol synthesis varies with 25-OH concentration. Fig. 1 shows the correlation between the inhibitions of these two processes. An inhibition of Ca^{+2}-dependent aggregation is apparent when cholesterol synthesis is inhibited by as little as 25%. A 60-70%

TABLE I

Effect of 25-OH Cholesterol and Compactin on Aggregation, Fusion, Sterol Synthesis, and the Sterol/ Phospholipid (PL) Molar Ratio

Treatment	Aggregation	Fusion	Sterol synthesis	Sterol/PL total membranes	Sterol/PL plasma membrane
	% control	% control	% control		
LDM	100	100	100	0.20 ± 0.03 (9)	0.52 ± 0.03 (3)
LDM + 25-OH cholesterol (1 μg/ml) at 5 h	0 ± 3.5 (3)	1.0 ± 1.7 (3)	12.6 ± 0.02 (2)	0.15 ± 0.03 (5)	0.32 ± 0.07 (2)
LDM + 25-OH cholesterol (1 μg/ml) at 47 h	10.1 ± 8.5 (5)	7.6 ± 2.3 (2)	29.9 ± 12.1 (10)	0.24 ± 0.03 (3)	0.59 ± 0.17 (3)
LDM + compactin (1 μg/ml) at 5 h	4.2 ± 1.2 (2)	ND*	29.2 ± 7.7 (3)	ND*	ND*

11-d chick primary myogenic cells cultured in LDM lacking calcium. 25-OH or compactin was added either 5 or 47 h after seeding. At 52 h (the optimum time for aggregation), cells were assayed for aggregation, [¹⁴C]acetate incorporation into sterol (2-h pulse) or sterol:phospholipid molar ratio. Fusion was scored at 72 h, i.e., 20 h after Ca^{+2} (1.8 mM) was added at 52 h. Aggregation was monitored in a suspension assay with cells detached from tissue culture plates with EDTA (13). The percent of cells in aggregates of two or more as judged by phase microscopy was scored after a 20-min incubation in buffer containing Ca^{+2} at 37°C on a rotary shaker. Fusion was scored as the percent of total nuclei in myotubes. The percent of cells in aggregates and the percent fusion for control cultures was 22.0 ± 4.5% and 22.4 ± 5.9, respectively. The numbers in parentheses denote the number of independent determinations.
* Not determined.

inhibition of sterol synthesis coincides with a complete inhibition of aggregation. However, there is no detectable change in the sterol:phospholipid ratio at a time interval and 25-OH dose that inhibit aggregation 90% (Table I). Thus, the addition of 25-OH inhibits aggregation appreciably before the cholesterol level is detectably reduced. There is also no effect, over a time interval sufficient to inhibit sterol synthesis and aggregation, on cell morphology or on the synthesis rates of phospholipid, triglyceride, glycoprotein, or total protein (data not shown).

Restimulation of Sterol Synthesis or Addition of Exogenous Cholesterol Restores Aggregation

The inhibition of cholesterol synthesis can be reversed by exchanging the medium containing compactin for conditioned medium lacking inhibitor or by adding mevalonic acid (the product of the inhibited hydroxymethylglutaryl-CoA reductase reaction) to compactin-treated cultures. These manipulations also reverse the inhibitory effect on aggregation. Fig. 2 shows the incubation time required to restore aggregation and cholesterol synthesis. When compactin is removed, restimulation of [^{14}C]acetate incorporation into sterol precedes, by a few hours, the restimulation of aggregation. Both are fully restored within 22 h.

FIGURE 2 Restimulation of sterol synthesis and aggregation by addition of mevalonate and by removal of compactin. Compactin (1.0–1.76 μg/ml) was added 5 h after seeding. At the indicated times, before the aggregation assay was initiated at 52 h ($t = 0$), 2 mg/ml mevalonolactone was added to the plates, or the medium containing compactin was replaced with inhibitor-free conditioned medium from parallel cultures. To measure the rate of sterol synthesis, cultures from which compactin had been removed were incubated with [^{14}C]acetate (0.66 Ci/ml) for 2 h before being harvested for analysis of labeled sterol at 53 h (O). Myoblasts were harvested and assayed for aggregation at 52 h after either removal of compactin (●) or addition of mevalonate (▲). Each point is the average of two to four experiments (four to eight determinations) with standard deviations. Control cultures were grown in LDM without compactin; the mean percent aggregation for controls was 40 ± 10%.

The inhibitory effect of 25-OH or compactin on aggregation can also be overcome by adding cholesterol, either as liposomes (cholesterol:lecithin, 1:1) or as LDL. Preparations of liposomes (1 μmol cholesterol/ml) or LDL (48–82 μg/ml) can restore aggregation to 70–80% of control (no compactin) when added as little as 4 h before the assay of aggregation and 100% when added 6 h before the assay (Fig. 3).[2, 3] This period of time is insufficient to raise the sterol:phospholipid ratio significantly above that of the compactin inhibited cells (Fig. 3). The absolute sterol mass per cell increases 20% after 6 h of exposure to LDL, but it would have to increase 63% to reach the control level. These observations also point to an apparent lack of correlation between myoblast aggregation and the ratio of sterol:phospholipid in their membranes.

A Comparison of the Effect of Cycloheximide, Tunicamycin, and Compactin on Ca^{+2}-mediated Aggregation

When myoblasts are incubated with 10 μg/ml cycloheximide, protein synthesis terminates within 10 min. The inhibition of glycoprotein synthesis by 2 μg/ml tunicamycin is much slower. The half-time for the inhibition of the rate is 1.5 h (data not shown). Fig. 4 shows that Ca^{+2}-mediated aggregation activity decays rapidly when the cells are cultured with 10 μg/ml cycloheximide or 2 μg/ml tunicamycin ($t_{1/2}$ is 4–6 h). In contrast to these inhibitors, the half-time for the inhibition of aggregation by 0.66 μg/ml compactin is only 1 h. Thus, the effect of
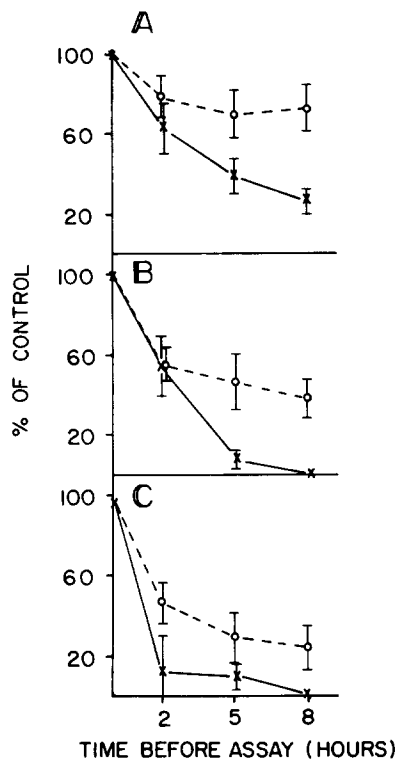
FIGURE 1 Dose dependence of the time-course of 25-OH mediated inhibition of aggregation and cholesterol synthesis. Primary cultures of chick myoblasts were plated at a density of 1.26 × 10^4 cells per cm^2 on 100-mm plates for aggregation and on 60-mm plates for [^{14}C]acetate incorporation for the experiments in this and all subsequent figures. At the times indicated, 25-OH was added to cultures at 0.31 μg/ml (A), 0.63 μg/ml (B), and 1.26 μg/ml (C). To measure the rate of sterol synthesis, cultures were pulsed for 2 h with 0.66 μCi/ml [^{14}C]acetate before harvesting for analysis of labeled sterol at 53 h (O). Myoblasts were harvested with EDTA and were assayed for aggregation at 52 h (X). The data, expressed as percent of control (i.e., no inhibitor), are the average of four to nine determinations with the standard deviations. The mean percent aggregation for control cultures was 24 ± 6%.
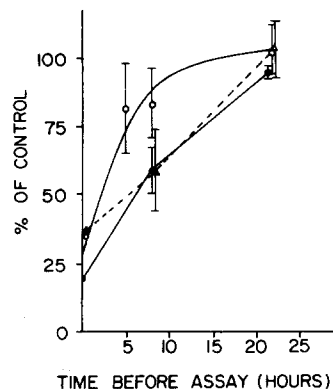
[2] Lipid vesicles composed solely of egg lecithin do not restore aggregation activity even after 20-h incubation with compactin-treated cells. Cholesterol-lecithin (1:1) vesicles added to compactin-free cultures do not enhance the Ca^{+2}-dependent aggregation above the control.
[3] The reversals by cholesterol, mevalonate, and inhibitor removal are also seen in assays of fusion as well as aggregation (reference 9 and Krussman and Horwitz, unpublished data).

inhibitors of sterol synthesis on aggregation is faster than the effect of tunicamysin or cycloheximide on inhibiting glycoprotein synthesis.

DISCUSSION

We have shown that the sterol synthesis inhibitors, 25-OH and compactin, inhibit myoblast fusion by interfering with myoblast recognition, i.e., rapid calcium-mediated aggregation. This confirms our previous, preliminary observation (9, 14).

There are many potential mechanisms by which these agents could inhibit recognition and fusion. One class includes effects of these agents unrelated to the production of sterol, i.e., interaction with cellular sites other than those on hydroxymethylglutaryl-CoA reductase or inhibition of dolichol synthesis. The former appears unlikely because compactin and 25-OH bear no structural resemblance yet produce a similar effect at the low, microgram concentrations required to inhibit HMG-CoA reductase. The synthesis of dolichol, the lipid intermediate involved in oligosaccharide transfer to protein, shares a common pathway with cholesterol; however, we have demonstrated with the L_6 myoblast cell line that the glycosylation of proteins does not appear to be inhibited substantially by treatment with 25-OH at levels that depress sterol synthesis >90% (5). This conclusion is supported by experiments with mineral oil-induced plasmacytoma 104E cells in which the incorporation of
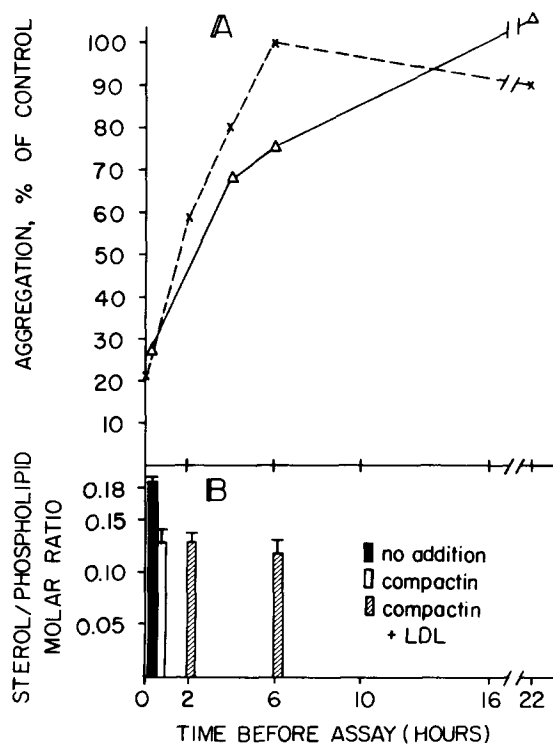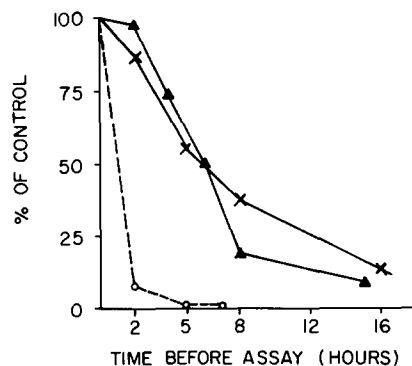

FIGURE 4 Comparison of the time-courses of inhibition of aggregation by cycloheximide, tunicamycin, and compactin. At the indicated times before aggregation assay was initiated at 52 h ($t = 0$), chick myoblast cultures received 10 μg/ml cycloheximide (X) 2 μg/ml tunicamycin (▲), or 0.66 μg/ml compactin (O). Each value on the compactin and cycloheximide curves is the average of two experiments (four determinations). The data on the tunicamycin curve is the composite of four experiments. Control cultures were grown in LDM without compactin. The mean percent aggregation of control cultures was 40 ± 5%. The average standard deviation for all three curves is ±4.5%.

$[^{14}C]$acetate into dolichol is unaffected by treatment with 25-OH at levels that inhibit sterol synthesis at least 90% (10).

The cholesterol requirement can be satisfied by restimulating de novo synthesis or by providing cholesterol exogenously either in LDL or in liposomes. The slower reversal kinetics observed using inhibitor removal or mevalonate addition is likely because of the requirement of several hours to activate the endogenous pathways. The observation that exogenous cholesterol completely restores the aggregation activity is the best argument for a link between the supply of cholesterol and the recognition process. That addition of cholesterol can reverse the inhibition by compactin as well as by 25-OH suggests that the rapid restoration of aggregation is not caused by competition between the two sterols for binding sites on the plasma membrane or on the reductase.

A second class of mechanisms addresses the issue of cell toxicity. This also appears unlikely because over the time scale of these experiments the 25-OH- and compactin-treated cells are intact by morphologic criteria, continue to synthesize total protein, glycoprotein, and nonsterol lipid at the control rate, and rapidly reverse the inhibition of Ca^{+2}-mediated aggregation upon addition of exogenous cholesterol.

The parameter with which our aggregation data appear to correlate most closely is the availability of sterol. One consequence of a decreased sterol production is a reduction in the levels of membrane cholesterol. The consequent alterations expected in the physical state of the membrane provide an attractive mechanism for the inhibition. Our data do not generally support this hypothesis because the bulk levels of membrane sterol do not correlate with changes in aggregation. Aggregation activity can be nearly totally blocked without a detectable change in the sterol:phospholipid ratio of either total cellular lipids or plasma membrane lipids. Furthermore, the activity can be restimulated at least fivefold without raising the ratio of total sterol/phospholipid. The aggregation also appears insensitive to the absolute sterol level per cell, which is 98% of control after 5-h of incubation with compactin and does not return to control levels in the time that LDL completely reverses the inhibition of aggregation activity when added to cells with a lowered sterol level.

FIGURE 3 (A) Restimulation of Ca^{+2}-mediated aggregation in compactin-treated cells (X) and liposomes (Δ) and (B) the effect of compactin and LDL on the sterol:phospholipid ratio. Compactin (1.1–2.2 μg/ml) was added at 5 h after seeding. At the indicated times before the aggregation assay was initiated at 52 h ($t = 0$), LDL (24–28 μg/ml) or liposomes (0.1–1.0 μmol cholesterol/ml) were added to some of the cultures. After 52 h in culture, the cells were harvested and assayed for Ca^{+2}-mediated aggregation or harvested for analysis of sterol and phospholipid content. Each point is the average of two to eight experiments. The average standard deviation is 13%. Control cultures were grown in LDM without compactin. The mean percent aggregation for control cells was 34 ± 6%.

The data do not, however, exclude the existence of a local cholesterol requirement. In this view, the unit with aggregation activity would be influenced by a domain of cholesterol that is the primary site to be depleted and replenished as sterol availability changes. Though unprecedented in animal cells, one such domain might be that used for the maintenance of existing recognition molecules or that used for the assembly or insertion of new molecules into the plasma membrane. In a membrane presumed to be fluid, these domains would necessarily be out of equilibrium with the bulk lipid. A cholesterol dependency for maintenance of the recognition activity would require that after synthesis, assembly, and insertion of the recognition protein, a continued local supply of cholesterol prevents enhanced shedding, degradation, or inactivation. A cholesterol requirement for assembly follows from the observation that the aggregation response to the addition of compactin is faster than the response to the inhibition of protein synthesis.[4] This suggests that the effect of cholesterol availability on aggregation is not mediated by a change in the rate of synthesis of the recognition protein.

One attractive hypothesis is that the assembly of the constituents to form the recognition activity is sensitive to the availability of cholesterol. A sizable intracellular precursor pool, like that described for acetylcholine receptors in chick myogenic cells (6), may also exist for the recognition protein. The protein may then partition into cholesterol-enriched regions of the plasma membrane, i.e., regions whose formation requires a ready supply of cholesterol. Alternatively, the intracellular membrane vesicles containing the nascent protein may be cholesterol rich.

Finally, we cannot exclude the hypotheses that propose a secondary, nonstructural metabolic perturbation. In this view, the recognition protein itself would have no direct dependence on the availability of cholesterol. Instead, a continuous source of cholesterol would be required to maintain other function(s) within the cell upon which the expression of the fusion activity (and perhaps other differentiated functions) depend. Further dissection of the cholesterol sensitivity of the myoblast recognition and fusion process awaits the development of suitable

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[4] The rapid decay of aggregation activity that results from pretreatment of myoblasts with cycloheximide or tunicamycin ($t_{1/2} \simeq 5$ h) indicates mediation by a recognition protein whose turnover is rapid (14). The half-life for the recognition protein is not necessarily equivalent to the half-life of aggregation activity because cooperation of several individual protein molecules may be required for unit activity. Thus, a half-life of 5 h is a minimum estimate for the protein itself.

methods for identifying the unit with aggregation activity. The present study demonstrates that (a) a surface membrane activity is highly sensitive to cholesterol availability and that (b) cholesterol appears to be required for assembly of the aggregation component(s) into the plasma membrane or for maintenance of the plasma membrane aggregation activity.

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