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Signal Transduction via Glycosyl Phosphatidylinositol-anchored Proteins in T Cells Is Inhibited by Lowering Cellular Cholesterol*

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Glycosylphosphatidylinositol (GPI)-anchored proteins can deliver costimulatory signals to lymphocytes, but the exact pathway of signal transduction involved is not yet characterized. GPI-anchored proteins are fixed to the cell surface solely by a phospholipid moiety and are clustered in distinct membrane domains that are formed by an unique lipid composition requiring cholesterol. To elucidate the role of membrane lipids for signal transduction via GPI-anchored proteins, we studied the influence of reduced cellular cholesterol content on calcium signaling via GPI-anchored CD59 and CD48 in Jurkat T cells. Lowering cholesterol by different inhibitors of cellular cholesterol synthesis suppressed calcium response via GPI-anchored proteins by about 50%, whereas stimulation via CD3 was only minimally affected (<10%). The decrease in overall calcium response via GPI-anchored proteins was reflected by inhibition of calcium release from intracellular stores. Cell surface expression of GPI-anchored proteins was not changed quantitatively by lowering cellular cholesterol, and neither was the pattern of immunofluorescence in microscopic examination. In addition, the distribution of GPI-anchored proteins in detergent-insoluble complexes remained unaltered. These results suggest that cellular cholesterol is an important prerequisite for signal transduction via GPI-anchored proteins beyond formation of membrane domains.

Activation of lymphocytes is the first step in any immune reaction. Apart from the specific interaction of major histocompatibility complex-coupled antigens with the T cell receptor complex, costimulatory factors are effective modulators of the immune response. Several glycosylphosphatidylinositol (GPI)[1]-anchored proteins can provide costimulatory signals to T cells (1–5). On human lymphocytes, CD59 (5, 6), CD55 (decay accelerating factor (7)), CD52 (8), and CD73 (9, 10) were shown to be involved in activation. On murine T cells, evidence for signal-transducing GPI-anchored proteins include Ly-6 (11–14), the major histocompatibility complex Ib molecule Qa-2 (15, 16), Thy-1 (17, 18), and sgp60, the murine analogue to CD48 (19–22). A variety of responses could be elicited by cross-linking of different GPI-anchored proteins on T lymphocytes, which included early events such as rise in intracellular calcium (6, 10, 11, 16), and protein tyrosine phosphorylation (1, 4, 23, 24). In addition, several late events have been observed as interleukin-2 production and proliferation (6, 7, 9, 11–13, 15, 16, 19, 21). Thus, GPI-anchored proteins may play a role in modulating the immune response.

GPI-anchored proteins are linked to the plasma membrane by a phospholipid moiety residing in the outer leaflet of the lipid bilayer (25). Since GPI-anchored proteins have no direct connection to the cytoplasm, the mechanism by which signal transduction can occur is not yet elucidated. However, GPI-linked proteins are clustered in membrane domains (26–28) that may be involved in T lymphocyte activation by mediating the association with Src family tyrosine kinases $p56^{lck}$ and $p59^{fyn}$ (1, 5, 26, 28–31). GPI-anchored protein complexes exhibit a particular lipid composition enriched in cholesterol and sphingolipids (32) that appears to be important for their clustering in living cells and the insolubility of GPI-anchored protein complexes in mild non-ionic detergents *in vitro* (32–35). Cellular transport and function of GPI-anchored folate receptor and alkaline phosphatase was abolished by sequestrating plasma membrane cholesterol by specific detergents (35–38). Accordingly, exogenously added CD59 was prevented from entering GPI complexes by filipin (5), and internalization of endogenous CD59 was partially inhibited by nystatin in lymphocytes (39), which suggests that membrane cholesterol plays an important role for the distribution and function of GPI-anchored proteins.

Here we studied the influence of membrane cholesterol on signal transduction via GPI-anchored CD59 and CD48 in T cells. To this end, cellular cholesterol content was reduced by culture in the presence of cholesterol synthesis inhibitors. Subsequently the rise in cytoplasmic calcium concentration was analyzed following cross-linking of GPI-anchored proteins or stimulation via the T cell receptor-CD3 complex, which served as a control. The calcium response was chosen for quantitative analysis of signal transduction since this parameter can be measured with high reliability. We show that cholesterol lowering particularly inhibits signal transduction via GPI-anchored proteins, whereas no changes were found in the distribution of these proteins in detergent-insoluble membrane domains.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All reagents were obtained from Sigma unless stated otherwise. Antibodies were used as follows: mouse monoclonal antibodies MEM-43 (IgG2a), MEM-43/5 (IgG2b), MEM-125 (IgM), MEM-129 (IgM, all four CD59), MEM-102 (IgG1, CD48), MEM-57 (IgG2a), MEM-92 (IgM, both CD3; all generated in the lab of

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[1] The abbreviations used are: GPI, glycosylphosphatidylinositol; GAM, goat anti-mouse; PI-PLC, phosphatidylinositol-specific phospholipase C; TCR, T cell receptor; FR, fluorescence ratio.

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Václav Hořejší and partly obtained from Monosan, Uden, The Netherlands), BRIC 216 (IgG1, CD55) (Haran Sera-Lab, Crawley Down, Sussex, UK), OKT3 (IgG2a, CD3) (Ortho Pharmaceuticals, Raritan, NJ), monoclonal antibody AAA6 (IgG1, CD147), and monoclonal antibody 0662 (IgG3, CD99; kindly provided by O. Majdic and A. Bernard, respectively); $F(ab')_2$-fragments of goat-anti-mouse (GAM) IgG (Sigma or Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), fluorescein isothiocyanate-labeled $F(ab')_2$-fragments of GAM Ig (Dako, Glostrup, Denmark), tetramethylrhodamine isothiocyanate-labeled $F(ab')_2$-fragments of GAM IgG (Accurate Chemical & Science Corp., Westbury, NY), GAM IgG labeled with horseradish peroxidase (Bio-Rad).

Cell Culture and Lipid Modifications—The human T cell line Jurkat E6–1 (American Type Culture Collection, Rockville, MD) was grown under standard conditions in RPMI 1640 medium supplemented with 10% heat-inactivated bovine calf serum (HyClone, Logan, UT), penicillin/streptomycin (50 units/ml and 50 μg/ml, respectively, Life Technologies, Inc.), and 2 mM glutamine (Life Technologies, Inc.) at 37 °C in humidified atmosphere in the presence of 5% CO_2. For modifications of cellular lipids, cells were incubated for 3 days in serum-free Iscove's modified Dulbecco's medium (Life Technologies, Inc.) supplemented with 0.4% (w/v) bovine serum albumin (fraction V), 1 mg/liter transferrin, 8.1 mg/liter monothioglycerol, 2 mM glutamine, and antibiotics as above (40). Cellular cholesterol synthesis was blocked by addition of 2 μM lovastatin (Merck), an inhibitor of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase, or squalestatin 1 (50 μg/ml) (Glaxo Wellcome), an inhibitor of squalene synthase, both generous gifts from the respective companies. The drugs were added to the culture medium from stock solutions in double distilled water (for squalestatin), or dimethyl sulfoxide (maximal solvent concentration 0.2%), respectively. Dimethyl sulfoxide was used as solvent control instead of lovastatin and had no effect on signal transduction (not shown). Sodium mevalonate was generated from mevalonolactone (Fluka Chemie AG, Buchs, Switzerland) by hydrolysis in NaOH. Cell viability was >90% as determined by trypan blue exclusion, and since INDO-1 also served as a vital dye only living cells were assessed with respect to their calcium response.

Quantitation of Calcium Response—Jurkat cells were labeled with the fluorescent Ca^{2+} indicator indo-1-AM (2 μM) (Molecular Probes, Inc., Eugene, OR) by incubation at 37 °C for 30 min in serum-free culture medium. Cells were primed with about 10 μg of antibodies to GPI-anchored proteins or Hanks'-buffered salt solution including 1% bovine serum albumin for 20 min followed by a 7 min preequilibration period at 37 °C. Subsequently, measurement of $[Ca^{2+}]_i$ by flow cytometry was started at 37 °C constant temperature, and after 60 s 20 μg cross-linking $F(ab')_2$-fragments of GAM IgG were added and measurement continued for another 5 min unless stated otherwise. For CD3 stimulation 2 μg of OKT3 was added directly without cross-linking. Cross-linking did not result in a strong calcium response in any OKT3 concentration with absence of calcium response by adding the primary antibody solely. Flow cytometric analysis was performed on a FACStar^{plus} (Becton Dickinson, San Jose, CA) using excitation by argon laser with 50-milliwatt multiline UV, emission 530 nm (Fl1, calcium-free indo-1) and 395 nm (Fl2, calcium bound form). The fluorescence ratio Fl2/Fl1 (FR), which is a direct estimate of the cytoplasmic calcium concentration (41), was computed in real time by a pulse processing unit and expressed as arbitrary units. The FR of the unstimulated control (FRus) was set to about 200 arbitrary units. For quantitation of stimulation, maximal FR was measured at 1–3 min after adding the stimulating antibody. Since CD3 stimulation of cells often induced a calcium response somewhat higher than stimulation via GPI-anchored proteins, results from modified cells were usually expressed as percentage of that achieved with the solvent control to allow comparability: relative stimulation in percent of control was calculated as (FRs - FRus)/(FRco - FRus) × 100%, with FRco representing FR of the solvent control, and FRs representing FR of the sample. Data are presented as means ± S.D. unless stated otherwise.

Release of Calcium from Intracellular Stores—Cells were prepared for stimulation of calcium response as described above. After preequilibration, SKF 96365 hydrochloride (Calbiochem) was added at optimal concentration (100 μM) to completely inhibit calcium entry from the medium (42). Two min afterward, cells were stimulated by antibodies as described, and changes in cytoplasmic calcium concentration were determined by flow cytometry over a 3-min period.

PI-PLC Treatment—10^6 cells were treated with 0.5 units of phosphatidylinositol-specific phospholipase C (PI-PLC) (Boehringer Mannheim) for 60 min at 37 °C in Hanks'-buffered salt solution including 0.5% bovine serum albumin. Cells were simultaneously loaded with INDO-1 for subsequent analysis of calcium response.

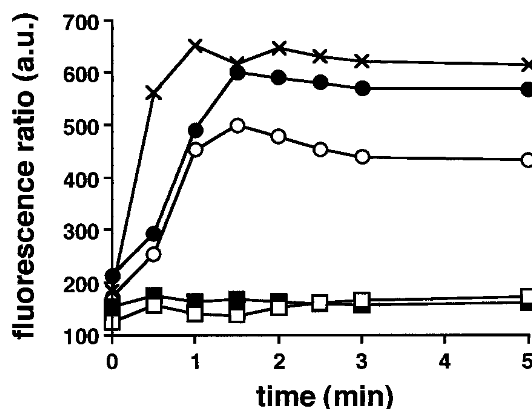

FIG. 1. **Calcium response after stimulation via GPI-anchored proteins.** Jurkat T cells were stimulated at time 0 via CD59 (MEM-43; ●) and CD48 (MEM-102; ○), or via CD3 (OKT3; ×) as positive control. CD147 (AAA6; ■) and CD99 (0662; □) served as negative controls. Original tracings of INDO-1 fluorescence ratio measured by flow cytometry are given. Expression of surface molecules as estimated by immunofluorescence (in arbitrary units): 176 (CD59, MEM-43), 67 (CD48, MEM-102), 119 (CD3, OKT3), 245 (CD147, AAA6), 204 (CD99, 0662), and 4 (phosphate-buffered saline control).

Analysis of Detergent-insoluble Complexes—Cells were washed three times in Hanks'-buffered salt solution and lysed for 30 min on ice with lysis buffer containing 20 mM Tris-HCl, pH 8.2, 140 mM NaCl, 1% Nonidet P-40 (Pierce), 10 μg/ml aprotinin (Bayer, Leverkusen, Germany), 5 mM iodoacetamide, 10 μg/ml leupeptin, and 0.4 mM Pefabloc (both Boehringer Mannheim), 1 μM pepstatin, and 0.1 mM N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone, N-CBZ-L-phenylalanine chloromethyl ketone, and quercetin. Lysates were then applied to Sepharose 4B minigel filtration columns as described earlier (26) with fractions 4 and 5 referring to the void volume. Aliquots of the fractions 3 to 11 were separated by 12% non-reducing SDS-polyacrylamide gel electrophoresis (43) and blotted on nitrocellulose membrane (Hybond ECL, Amersham International, UK) using a semidry blotting system (C.B.S. Scientific Co., Inc., Del Mar, CA). Membranes were developed according to standard Western blotting procedures, and detection was performed by the chemiluminescence system from Boehringer Mannheim.

Immunofluorescence Analysis and Determination of Cholesterol Content—Indirect immunofluorescence for cell surface expression of GPI-anchored proteins on unfixed cells was performed by standard procedures with tetramethylrhodamine isothiocyanate-labeled second antibody for flow cytometric analysis. For microscopic examination, cells were first fixed for 20 min in 4% formaldehyde followed by 0.1 M glycine before immunofluorescence staining. Photographs were taken from immunofluorescence with tetramethylrhodamine isothiocyanate-labeled GAM Ig, since fluorescein isothiocyanate fluorescence faded before adequate exposure of the film (Kodak EPH1600) could be achieved. For determination of cholesterol content, 3 × 10^7 cells were twice extracted with chloroform/methanol (2:1). Dried lipid extracts were dissolved in ethanol, and cholesterol was quantified by a commercial enzymatic colorimetric test according to the manufacturers instructions (Boehringer Mannheim).

RESULTS

Calcium Signaling via GPI-anchored Proteins—Cross-linking of GPI-anchored proteins CD59 or CD48 resulted in a marked rise in intracellular calcium as measured by flow cytometry (Fig. 1). The signal via CD59 quantified by INDO-1 fluorescence ratio was consistently at least half of that elicited by CD3, which was used as a positive control. No calcium signal was obtained by cross-linking of antibodies directed to CD147 or CD99 molecules, which were well expressed on the Jurkat cell line used, or with isotype controls (not shown). Addition of CD59 or CD48 antibodies without cross-linking had no direct effect on the cytoplasmic calcium concentration (not shown).

To test for the specificity of the calcium response for GPI-anchored proteins, cells were treated with PI-PLC to cleave the glycoproteins from their phospholipid anchor. Following treat-

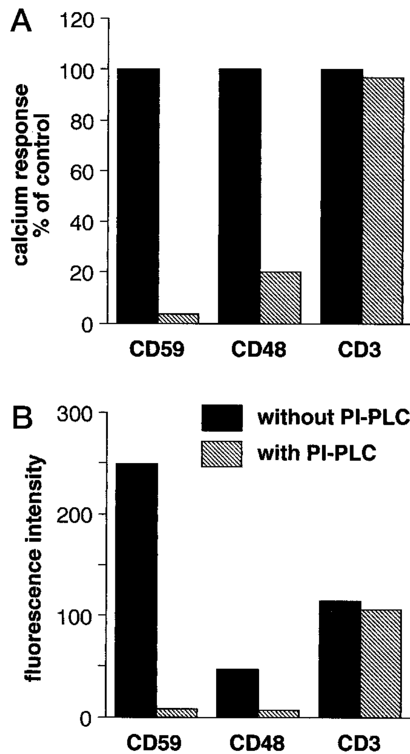

FIG. 2. **PI-PLC treatment abolishes expression and calcium responses via GPI-anchored proteins.** *A*, calcium response elicited in Jurkat cells pretreated with or without PI-PLC and subsequently stimulated via CD59 (MEM-43), CD48 (MEM-102), or CD3 (OKT3). Calcium responses without enzyme pretreatment were set to 100%. *B*, analysis of surface expression of GPI-anchored proteins (CD59, CD48) and CD3 after pretreatment with or without PI-PLC. Results from one of two independent experiments are depicted.

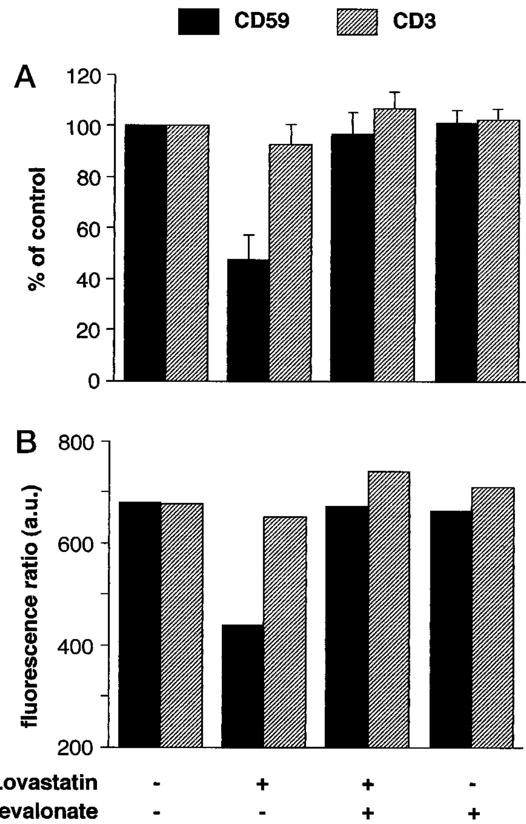

FIG. 3. **Inhibition by lovastatin of CD59 but not of CD3 induced calcium response.** Jurkat cells were incubated for 3 days in serum-free medium with lovastatin (2 μM), an inhibitor of cellular cholesterol synthesis, and mevalonate (7.5 mM) to rescue from this inhibition, as indicated. Subsequently, cells were stimulated via CD59 (MEM-43) or CD3 (OKT3). *A*, calcium responses are expressed as percentage of the response elicited in untreated cells (solvent control = 100%). Means ± S.D. from eight independent experiments are given. *B*, calcium responses expressed as INDO-1 fluorescence ratio (in arbitrary units (*a.u.*)) from a single experiment. Note that the absolute rise in cytosolic calcium in the control (*i.e.* without addition of lovastatin or mevalonate) was almost identical with CD59 and CD3 in this experiment.

ment, CD59 or CD48 could no longer be detected on the cell surface and the calcium signal was essentially abolished, whereas surface expression and calcium response elicited via CD3 remained unchanged (Fig. 2, *A* and *B*).

Cholesterol Depletion Inhibits Calcium Signaling via GPI-anchored CD59—Cellular cholesterol content was lowered by blocking endogenous cholesterol biosynthesis by lovastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase catalyzing the reaction to mevalonate (44). Treatment of cells with 2 μM lovastatin for a 3-day period decreased cellular cholesterol content by 28% compared with the solvent control (1.71 ± 0.09 nmol *versus* 1.24 ± 0.15 nmol/10^6 cells). Under the same conditions, lovastatin inhibited CD59-driven calcium response by 52 ± 9%, whereas the response via CD3 was only minimally affected (7 ± 8% inhibition; Fig. 3, *A* and *B*). The inhibition by lovastatin could be reversed by added mevalonate (7.5 mM) showing that no unspecific toxicity was responsible for the drug effect. Accordingly, lovastatin did not alter calcium response of Jurkat cells when added to serum-supplemented medium (91 and 97% of solvent control for CD59 and CD3, respectively), presumably because of the presence of serum lipoproteins providing exogenous cholesterol (data not shown).

Lovastatin inhibits production of mevalonate that can be further processed not only to cholesterol but also to other lipophilic products (44) including farnesyl and geranylgeranyl moieties needed for prenylation of proteins, *e.g.* of the Ras family or G-protein γ subunits. To further prove lovastatin's inhibitory effect to be due to cholesterol lowering, squalestatin 1, an inhibitor of the squalene synthase, was also tested for its effect on signal transduction via GPI-anchored proteins. Squalestatin 1 blocks cholesterol biosynthesis at a step distal to mevalonate synthesis and, therefore, does not affect side products of the mevalonate pathway (45). Squalestatin 1 inhibited signal transduction via CD59 similarly to lovastatin (47 ± 14% inhibition; Fig. 4) with minimal effect on CD3 signaling (2 ± 3% inhibition). The associated block in cholesterol synthesis downstream from mevalonate could not be reverted by mevalonate but only by cholesterol.

Inhibition of Calcium Response by Cholesterol Lowering is Common to Different GPI-anchored Proteins and Is Not Due to Their Altered Surface Expression—The inhibitory effect of cholesterol depletion on calcium response is not restricted to CD59 but shared by CD48, another GPI-anchored protein (Fig. 5). Thus, inhibition by cholesterol depletion seems to be characteristic for signal transduction via GPI-anchored proteins.

Immunofluorescence analysis of lovastatin-treated cells revealed no alterations in cell surface expression of GPI-anchored proteins CD59 and CD48, and CD3 (Fig. 6). The expression of at least two different epitopes on CD59 (epitope 1: MEM-43; epitope 2: MEM-43/5, MEM-125 (46)) was unaltered upon blockade of cellular cholesterol synthesis, suggesting absence of gross conformational changes of the protein part of CD59 (Fig. 6). The markedly diminished surface fluorescence detected by monoclonal antibodies MEM-125, MEM-129 (CD59), and MEM-92 (CD3) compared with other antibodies of the same antigen is due to their different isotype (IgM).

Influence of Cholesterol Depletion on Calcium Release from Intracellular Stores—Transient release of calcium from intra-

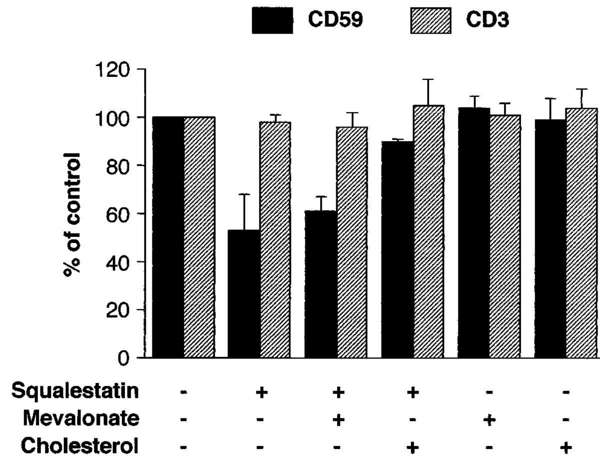

FIG. 4. **Inhibition by squalestatin of CD59 but not CD3 induced calcium response.** Jurkat T cells were incubated for 3 days in serum-free medium supplemented with squalestatin (50 μM), an inhibitor of cellular cholesterol synthesis, and mevalonate (7.5 mM), or cholesterol (25 μg/ml) to test for possible rescue from this inhibition, as indicated. Cells were stimulated for calcium response via CD59 (MEM-43) or CD3 (OKT3), and the response of the untreated control was set to 100%. Means ± S.D. from three independent experiments.

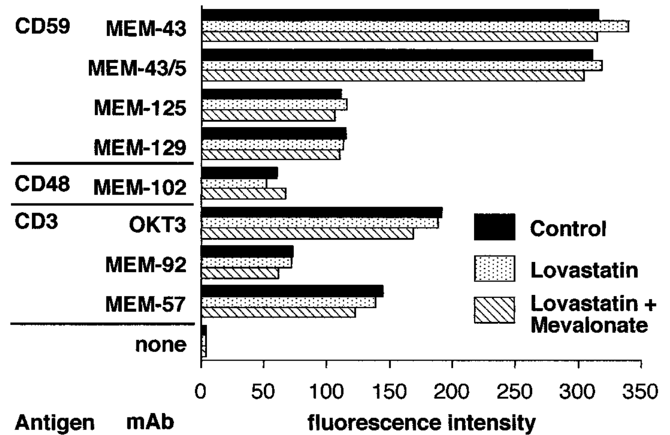

FIG. 6. **Cholesterol depletion does not alter surface expression of GPI-anchored proteins.** Jurkat cells were incubated with solvent (*black bars*), lovastatin (2 μM) (*speckled bars*), or lovastatin and mevalonate (7.5 mM) (*hatched bars*) for 3 days in serum-free medium. Cell surface immunofluorescence was determined using various antibodies to CD59 (MEM-43, MEM-43/5, MEM-125, MEM-129), CD48 (MEM-102), and CD3 (OKT3, MEM-92, MEM-57). Data from one of three independent determinations are shown.

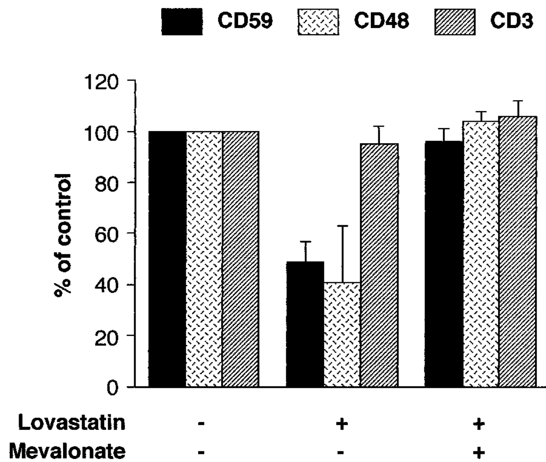

FIG. 5. **Inhibition by cholesterol depletion of calcium response via different GPI-anchored proteins.** Jurkat T cells were incubated for 3 days in serum-free medium containing 2 μM lovastatin and 7.5 mM mevalonate as indicated. Cells were stimulated for calcium response via CD59 (MEM-43), CD48 (MEM-102), or CD3 (OKT3), which served as a control. The response of the untreated control was set to 100%. Means ± S.D. from three independent experiments are depicted.

cellular stores into the cytoplasm precedes capacitative calcium entry from the environment leading to further activation of T lymphocytes (47, 48). To test whether inhibition of the release of intracellularly sequestered calcium underlies the total decrease in calcium response, cells were treated with SKF 96365 prior to stimulation. The maximal concentration of cytoplasmic calcium achieved following stimulation via GPI-anchored CD59 was decreased by about 40% after blockade of cholesterol synthesis (Fig. 7*A*). In contrast, stimulation via CD3 yielded no difference between lovastatin-treated and untreated cells (Fig. 7*B*).

Cholesterol Lowering Does Not Alter Clustering of GPI-anchored Proteins and Distribution in Detergent-insoluble Complexes—Cholesterol lowering may interfere with clustering of GPI-anchored proteins and distribution in detergent-insoluble complexes. Cells fixed in formaldehyde prior to indirect immunofluorescence to avoid artificial clustering by antibodies, showed a punctate pattern of CD59 and CD48 expression (Fig. 8, *A* and *C*) that was maintained after pretreatment of cells with lovastatin (Fig. 8, *B* and *D*). Investigating the impact of cholesterol depletion on the distribution of CD59 in large detergent-insoluble complexes, cells cultured with or without lovastatin were lysed in buffer containing non-ionic detergents Brij-58 or Nonidet P-40 and analyzed by size fractionation and Western blotting. Thereby most of GPI-anchored proteins CD59 and CD48 were found in large membrane complexes although after lysis with Nonidet P-40 a significant amount of CD59 was recovered in lower molecular weight fractions (Fig. 9). However, the distribution of GPI-anchored proteins was identical irrespective of whether cells have been cholesterol depleted or not. Thus, the influence of cholesterol depletion on signal transduction via GPI-anchored proteins seems not to be due to destruction of GPI-anchored protein complexes.

DISCUSSION

The obtained data show that cholesterol lowering in living cells by metabolic intervention effectively inhibits signal transduction via GPI-anchored proteins without changing their clustering or distribution in large detergent-insoluble complexes. In contrast, signaling via the T cell receptor (TCR)-CD3 complex was hardly affected by cholesterol deprivation.

Since GPI-anchored proteins lack a transmembrane domain, the clustering of these proteins to specific membrane regions seems to be prerequisite for signal transduction. Cholesterol is an essential component for the formation of these detergent-insoluble complexes (5, 35–37, 39) that mediate the association with possible signal-transducing molecules, *e.g.* Src family kinases $p56^{lck}$ and $p59^{fyn}$ (1, 29–31) or G-proteins (49). It has been shown that exogenously added fluorescent CD59 needs to aggregate into clusters before signal transduction is enabled (5). Lowering cholesterol by 60% in living epithelial cells by blocking its endogenous synthesis resulted in strong inhibition of folate uptake via the GPI-linked folate receptor along with variable differences in clustering of these receptors (50). In our study, reduction in lymphocyte cholesterol content by about 30% did not disclose any alterations in the distribution of GPI-anchored proteins when examined by immunofluorescence or gel filtration of membrane complexes. In addition, lowering cell cholesterol had no influence on overall cell surface expression of GPI-anchored proteins as quantitated by flow cytometry in contrast to previous publications (51–53). Though delipidation may cause some conformational changes in GPI-anchored

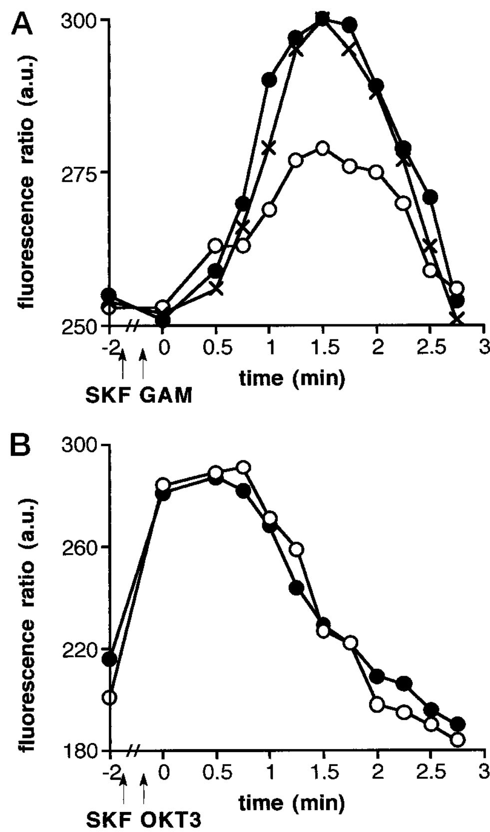

FIG. 7. **Cholesterol depletion inhibits release of calcium from intracellular stores when induced by GPI-anchored proteins but not after stimulation by CD3.** Cells were grown in serum-free medium for 3 days in the absence (●) or presence (○) of 2 μM lovastatin; a third sample of cells cultured in presence of lovastatin and 7.5 mM mevalonate was stimulated via CD59 to rescue from the lovastatin-induced inhibition (×). SKF 96365 (100 μM) was added to inhibit calcium entry and followed by GAM cross-linking antibody in the case of cells primed with anti-CD59 (MEM-43; *panel A*) or by antibody against CD3 (OKT3; *panel B*), respectively. Calcium response reflecting release of intracellular calcium stores was monitored for almost 3 min following stimulation. Data from one of three experiments are shown.

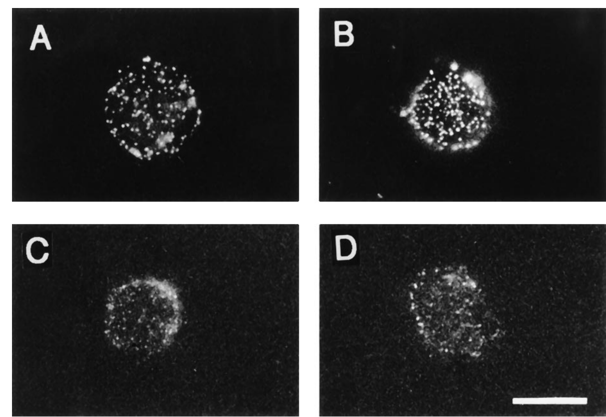

FIG. 8. **Cholesterol lowering fails to affect distribution of GPI-anchored protein on the cell surface.** Cells cultured for three days in serum-free medium with (*panels B* and *D*) or without (*panels A* and *C*) lovastatin were fixed with formaldehyde and subsequently stained for surface expression of CD59 (MEM-43; *panels A* and *B*) and CD48 (MEM-102; *panels C* and *D*) by indirect immunofluorescence using tetramethylrhodamine isothiocyanate-labeled goat-anti-mouse IgG. No fluorescence was detected on phosphate-buffered saline controls (not shown). *Bar*, 5 μm.

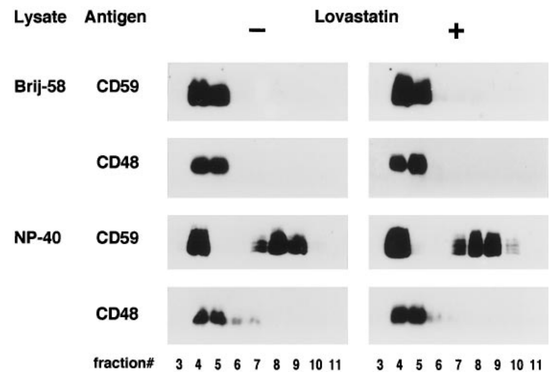

FIG. 9. **Cholesterol lowering fails to influence distribution of GPI-anchored proteins in detergent-insoluble complexes.** Jurkat T cells were incubated for 3 days in serum-free medium with (−) or without (+) 2 μM lovastatin as indicated. Cells were lysed in lysis buffer containing 1% of the non-ionic detergent Brij-58 or Nonidet P-40 (*NP-40*), respectively. Subsequently, detergent-insoluble complexes were separated by minigel chromatography with fractions 4 and 5 corresponding to the void volume and with IgM and IgG eluting in fractions 7 and 9, respectively (26). The proteins of each fraction were separated by non-reducing polyacrylamide gel electrophoresis and identified by Western blotting with antibodies against CD59 (MEM-43/5) or CD48 (MEM-102) as indicated. Results from one of three independent analyses are depicted.

proteins (54) this seems to be unlikely in our experiments, since antibodies against at least two different epitopes on CD59 (46) bound to cholesterol-depleted and control cells to a similar extent (Fig. 5). Thus, in contrast to rather harsh treatments with sterol-sequestrating detergents, which are able to destroy detergent-insoluble complexes *in situ* (5, 36, 37, 39), the signal-transducing function of these proteins seems to be particularly sensitive to a disturbed cholesterol availability.

Although GPI-anchored proteins partly share signaling events with the TCR, there may be unique steps in the signaling pathways for either type of activation. Signal transduction via the TCR-CD3 complex but also distinct signaling events induced via GPI-anchored proteins including interleukin-2 secretion, CD69 expression, and activation of nuclear factor-κB, require expression of the TCR-ζ chain (14, 55–58). On the other hand, induction of calcium response (55), activation of $p56^{lck}$, protein kinase C, and ERK-2, expression of CD25 (58), and inhibition of CD3-driven interleukin-2 production (57, 59) were shown to be independent of the expression of TCR-ζ. Furthermore, activation of murine T cells via GPI-anchored Thy-1 but not via CD3 requires expression of $p59^{fyn}$ (60). Thus, signaling via GPI-anchored proteins seems to differ from TCR/CD3 signaling with regard to the involvement of the TCR-ζ chain and particular Src protein kinases. Despite these differences, both signaling pathways were shown to interact with each other since adequate expression of GPI-anchored proteins is needed for signaling via the TCR-CD3 complex (61, 62). It appears from this that T cell activation via GPI-anchored proteins differs from TCR/CD3-driven activation, and each pathway may require components of the other for optimal stimulation (2, 61).

Our findings, that activation of calcium response in T cells via GPI-anchored proteins and the TCR is differentially affected by cholesterol depletion, supports the concept of separate signaling pathways to be involved. Moreover, since activation via CD59 and CD48 were suppressed to a similar extent, cholesterol lowering seems to impair signaling pathways common to GPI-anchored proteins. Absence of structural homologies between both GPI-linked molecules beyond the phospholipid anchor suggests that the GPI-anchor itself could play an important role in signal transduction via these proteins, as proposed previously by others (30, 61, 63, 64).

Rescue by appropriate downstream products of cholesterol synthesis proves that the drugs applied are actually effective via inhibition of cholesterol biosynthesis. Such inhibition of

cholesterol synthesis in the endoplasmic reticulum may directly interfere with more subtle associations of lipids and proteins during aggregation to detergent-insoluble complexes. How a disturbed lipid composition can alter the signal transduction process remains to be elucidated. Conceivably, spatial relationships between GPI-anchored proteins and candidate signal-transducing molecules like the Src family protein tyrosine kinases could play a role in this process as well as changes in lateral mobility of components within the complexes.

In conclusion, our data suggest that cholesterol lowering affects signal transduction via GPI-anchored proteins in a very early stage up to the release of calcium without apparent structural alterations. Thus, lipids may play an intriguing role in signal transduction via these surface molecules whose exact molecular mechanisms remain to be elucidated.

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