Ganglioside GM3 activates ERKs in human lymphocytic cells

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Abstract In this study we analyzed the signaling pathway triggered by GM3 in lymphoblastoid T-cells. In these cells, GM3 induced cPLA$_2$ activation, arachidonic acid release, and PKC-δ translocation. In order to clarify the upstream molecular signals triggered by GM3, we analyzed the activation of extracellular signal-regulated kinase (ERK)s, a downstream effector of Ras-regulated cytoplasmic kinase cascade. Our results showed that GM3 treatment led to rapid ERK phosphorylation in lymphoblastoid T-cells, as detected by anti-phospho-p44/42 MAP kinase. Similar findings were found in human peripheral blood lymphocytes. Moreover, we showed that GM3 specifically phosphorylated ERK-2, as revealed by anti-phosphotyrosine reactivity on both cell free lysates and ERKs immunoprecipitates. The role of the CD4 cytoplasmic domain in GM3-triggered signaling pathway was investigated using A2.01/CD4-cyt399 cells, which had been transfected with a mutant form of CD4 lacking the bulk of the cytoplasmic domain. In these cells GM3 induced cPLA$_2$ activation, arachidonic acid release, and PKC-δ translocation, but not CD4 endocytosis, indicating that the CD4 cytoplasmic domain plays a key role in GM3-triggered CD4 endocytosis and the GM3-triggered biochemical pathway is upstream of CD4 phosphorylation. These findings strongly suggest that GM3 triggers a novel signaling pathway involved in the regulation of cellular functions.—Garofalo, T., M. Sorice, R. Misasi, B. Cinque, V. Mattei, G. M. Pontieri, M. G. Cifone, and A. Pavan. **Ganglioside GM3 activates ERKs in human lymphocytic cells. *J. Lipid Res.* 2002.** 43: **971–978.**

Supplementary key words extracellular signal-regulated kinase • CD4 • lipid domains • glycosphingolipids

Gangliosides are sialic acid-containing glycosphingolipids that are present in all mammalian cells. Although their biological functions are not completely clarified, recent evidence has indicated that, by interacting with specific (glyco)proteins in glycosphingolipid-enriched microdomains, gangliosides are involved in cell-cell interaction, cell activation, and signal transduction (1–5). In addition, several lines of evidence have suggested the involvement of the glycosphingolipids in the regulation of cell proliferation (6–7) and/or cell death (8–9).

However, the molecular mechanisms of ganglioside-mediated cellular effects are not completely understood. Exogenous gangliosides, as amphiphilic molecules, are incorporated into cellular membranes, as demonstrated by an overall increase of the ion permeation across the plasma membrane and an enhanced polarizability of their hydrophobic region (10). Different gangliosides seem to act as modulators of the activity of specific surface receptors. Indeed, GM3 was able to regulate fibroblast growth factor (FGF) and epidermal growth factor (EGF) receptor activities, through the modulation of tyrosine-kinase activity (11–12). GM1 enhanced the action of nerve growth factor (NGF) by binding to Trk, the tyrosine kinase-type receptor for NGF (13). Furthermore, exogenous gangliosides may participate in the activation of multiple signal transduction pathways. GM1 induced early tyrosine phosphorylation of phospholipase Cγ-1 (14) and an increase in intracellular Ca^{2+} through a $p56^{lck}$-dependent pathway (15). Gangliosides play a role in regulating G-protein activity, since they can act as inhibitors of ADP-ribosyltransferases (16), which cause the functional uncoupling of G-proteins from receptors; in addition, gangliosides may control cell growth by modulating the activity of protein kinase C (PKC) (17). In favor of this possibility, it was shown that specific stimuli, able to activate PKC in neuronal cells, induced a change of plasma membrane ganglioside distribution (18) and/or content (19).

Moreover, in human glioma cells, gangliosides may trigger specific mitogenic signal transduction pathways, such as the activation of mitogen-activated protein kinase (MAPK) isoform extracellular signal-regulated kinase-2

Abbreviations: AA, arachidonic acid; AACOCF$_3$, trifluoromethylketone analog of arachidonic acid; cPLA$_2$, cytosolic phospholipase A$_2$; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinase; FGF, fibroblast growth factor; HRP, horseradish peroxidase; NGF, nerve growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PBL, peripheral blood lymphocytes; PC, phosphatidylcoline; PKC, protein kinase C.

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(ERK-2), which was shown to be involved in the GM1-mediated proliferation of U-1242 MG glioma cells (20). In addition, GM1 treatment leads to activation of $p70^{s6k}$, stimulating another distinct signaling pathway involved in the increase of DNA synthesis (20).

In human lymphocyte GM3, which is the major ganglioside constituent (21), specifically induced a rapid cytosolic phospholipase A_2 ($cPLA_2$) activation with consequent arachidonic acid release and PKC activation, which resulted in CD4 serine phosphorylation and endocytosis (22). CD4 down-modulation on T-cell plasma membrane is a well known molecular event triggered by different stimuli, through PKC activation, phosphorylation of serine residues in cytoplasmic tail of CD4 molecule, and dissociation of CD4-$p56^{lck}$ complex (23). In our previous report (22), we observed that GM3 effects were blocked by the MEK inhibitor PD98059 and hypothesized that $cPLA_2$ activation might be mediated by ERKs, a downstream effector of a Ras-regulated cytoplasmic kinase cascade, which includes RAF-1 and MEK (24). Therefore, in this study we analyzed the GM3 effect on MAP kinase activity and on the signaling pathway leading to CD4 endocytosis. With this aim, we used two different lymphoma-derived T-cell lines, A2.01/CD4-cyt399, that had been transfected with a mutant form of CD4 lacking the bulk of the cytoplasmic domain, and A3.01, which expressed wild-type CD4 (25).

MATERIALS AND METHODS

Cells

Human peripheral blood lymphocytes (PBL) were isolated from fresh heparinized blood by Lymphoprep (Nycomed AS Pharma Diagnostic Division, Oslo, Norway) density-gradient centrifugation and washed three times in phosphate-buffered saline (PBS), pH 7.4.

The lymphocytic cell line CEM (26), as well as CEM-derived A2.01/CD4-cyt399 and A3.01 (25), were grown in RPMI 1640 supplemented with 10% FCS, penicillin, streptomycin and, for A2.01/CD4-cyt399, 0.4–1.0 mg/ml G418 (Gibco, BRL, Paisley, Scotland) and used for experiments when they were growing exponentially.

Flow cytometry analysis of GM3-treated cells

Cells (1×10^6 in 1 ml of PBS) were incubated in the presence of 50 μM bisindolylmaleimide GF109203X, (Boehringer Mannheim, Milano, Italy) (27), or 10 μM rottlerin (Calbiochem, La Jolla, CA) (28). After a 15 min incubation, 50 μg/ml GM3 (Alexis, San Diego, CA), was added for 30 min at 37°C. Incubation with GM3 at 37°C did not affect the viability of the cells. At the end of the incubation time, the cells were washed with PBS before incubation with antibodies.

Cells either untreated or treated with the gangliosides in the presence or in the absence of the PKC inhibitors were stained directly using fluorescein conjugated monoclonal antibody OKT4 (Orthodiagnostic, Raritan, NJ), which recognizes the juxtamembrane epitope of CD4. After washing with PBS, cells were fixed in 2% formaldehyde in PBS. Green fluorescence intensity was analyzed on an EPICS profile flow cytometer (Coulter Electronics, Hialeah, FL). Vital cells were gated on the bases of forward angle and 90° light scatter parameters.

Scanning confocal microscopy

Cells (1×10^6 in 1 ml of PBS) were incubated with GM3, 50 μg/ml, for 5 min at 37°C. GM3 treated and untreated cells were then fixed with acetone-methanol 1:1 (v/v) for 10 min at 4°C. Cells were soaked in balanced salt solution (Sigma Chem. Co., St Louis) for 30 min at 25°C and incubated for 20 min at 25°C in the blocking buffer of 2% BSA in PBS containing 5% glycerol and 0.2% Tween 20. Cells were then labeled with anti-PKC-δ MAb (Santa Cruz Biotec., Santa Cruz, CA), for 1 h at 4°C. After three washes in PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 45 min at 4°C. The redistribution of PKC isozyme was analyzed by scanning confocal microscopy, using a Sarastro 2000 (Molecular Dynamics, Sunnyvale, CA) attached to a Nikon Optiphot microscope (objective PLAN-APO 60/1.4 oil) and equipped with argon ion laser (25 mW). FITC was excited at 488 nm and laser power was set at 1 mW. Images were collected at 512×512 pixels with voxel dimensions 0.08 μm (lateral), 0.49 μm (axial). After having been processed with routines for noise filtering, serial optical sections were assembled in Depth-Coding mode. Acquisition and processing were carried out using Image Space software (Molecular Dynamics).

Phospholipase A_2 (PLA_2) activity assay

Cells (1×10^6) were incubated with GM3 (50 μg/ml) for 1, 2, and 5 min at 37°C in RPMI 1640. Where indicated, the cells were treated with an inhibitor of $cPLA_2$, 10 μM $AACOCF_3$ (trifluoromethylketone) analog of arachidonic acid (Biomol, Plymouth Meeting, PA), for 1 h before GM3 addition. Incubation was stopped by centrifugation at 500 *g* for 1 min at 4°C. The cell pellets were resuspended in 250 mM Tris-HCl buffer pH 8.5, containing 10 μM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin, 5 μg/ml soybean trypsin inhibitor, 100 μM bacitracin, and 1 mM benzamidine. Cells were lysed by sonication and protein concentrations were determined using a Bio-Rad protein assay. Radiolabeled PC vesicles were prepared by sonication of the radiolabeled phospholipid L-3-phosphatidylcholine, 1-stearoyl-2-[1-^{14}C]arachidonyl (Amersham, Little Chalfont, UK) in 50 mM Tris-HCl buffer pH 8.5, containing fatty acid-free BSA (0.01%) in an ice bath (5 min, 5 W and 80% output). Vesicles were resuspended at 1 μM in the reaction buffer (50 mM Tris-HCl pH 8.5, 5 mM $CaCl_2$, 5 mM $MgCl_2$) with 60–100 μg whole cell lysate for 1 h at 37°C, and the reaction was stopped by the addition of 250 μl of chloroform-methanol-acetic acid (4:2:1, v/v/v). Phospholipids were extracted, dried under nitrogen, resuspended in 200 μl chloroform, and applied in duplicate to a silica gel TLC plate. Samples were chromatographed in chloroform-methanol-acetic acid-water (100:60:16:8, v/v/v/v) to separate the labeled product of PLA_2 activity, i.e., arachidonic acid (AA). The radioactive spots were visualized by autoradiography, scraped from the plate, and counted by liquid scintillation. PLA_2 activity was expressed as pmol AA produced/10^6 cells.

Analysis of ERK activation

Cells (1×10^6/ml) were incubated with 50 μg/ml GM3 or, as a control, with phorbol ester myristate acetate (PMA) (50 ng/ml) for 1, 2, and 5 min at 37°C, in serum-free RPMI 1640. The cells were washed twice with ice-cold PBS, resuspended in lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM dithiothreitol), and subjected to three rounds of freeze-thaw lysis. About 50 μg of extracted cell proteins were separated on 12% SDS-PAGE gels under reducing conditions, and proteins were transferred electrophoretically to nitrocellulose membrane. Non-specific binding sites were blocked with 10% non-fat dry milk in Tris-buffered saline-Tween 20 (0.3%) (20 mM Tris, 150 mM NaCl pH 7.6) for 1 h at room temperature and the blots were incubated overnight with the following antibodies: polyclonal anti-ERKs (K-23

Santa Cruz Biotechnology) or monoclonal anti-phospho-p44/42 MAP kinase (New England Biolabs, Inc.) under the same conditions. Bound antibodies were then visualized with HRP-conjugated anti-rabbit or anti-mouse IgG, and immunoreactivity assessed by chemiluminescence using the ECL detection system (Amersham, Buckinghamshire, UK). Manufacturer-specified protocols were used to strip the membrane to reprobe with monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology, Lake Placid, NY), followed by HRP-conjugated anti-mouse IgG. Densitometric scanning analysis was performed by Mac OS 9.0 (Apple Computer International), using NIH Image 1.62 software.

Alternatively, for the analysis on ERKs immunoprecipitates, cell free lysates from untreated human PBL, GM3-treated cells (5 min, 50 μg/10^6 cells/ml), and GM3-treated cells (5 min, 50 μg/10^6 cells/ml), previously incubated with 50 μM MEK inhibitor PD98059 (2'-amino-3′methoxyflavone; Calbiochem, La Jolla, CA) (29) (30 min), were washed twice with ice-cold PBS, incubated at 4°C for 10 min in RIPA buffer (PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mg/ml phenylmethylsulfonyl fluoride, 50 μg/ml aprotinin, 100 mM sodium orthovanadate), and then disrupted by repeated aspiration through a 21 gauge needle. Lysates were cleared by centrifugation at 15,000 *g* for 30 min at 4°C and protein concentration determined using BIORAD protein assay reagent. After preclearing, cell-free lysates normalized for proteins were incubated with polyclonal anti-ERKs (K-23 Santa Cruz Biotechnology) and then with protein G-sepharose beads. The mixtures were centrifuged and washed three times with 0.4 ml of the RIPA buffer. The pellets resuspended in loading buffer were resolved on 12% SDS-PAGE gels under reducing conditions and immunoreactivity was assessed as above.

RESULTS

GM3-induced CD4 down-modulation occurs only in cells expressing wild-type CD4

CD4 is an integral membrane glycoprotein and contains a cytoplasmic domain of 38 aminoacids (30). Since the three main phosphorylation sites of CD4 are localized in the cytoplasmic region (31), we investigated the role of this domain in GM3-induced CD4 endocytosis using two transfected cell lines derived from the CEM cell line. We used A2.01/CD4-cyt399, which have been transfected with a mutant form of CD4 lacking the bulk of the cytoplasmic domain (cytoplasmic amino acids 399–433), in comparison with A3.01, which express wild-type CD4. Cytofluorimetric analysis, performed using the OKT4 antibody, revealed that incubation of A2.01/CD4-cyt399 cells with GM3 (50 μg/ml/10^6 cells) did not significantly affect the CD4 staining (**Fig. 1A**, 1B), revealing that in A2.01/CD4-cyt399 the GM3-induced CD4 down-modulation did not occur.

In contrast and in agreement with our previous results on human PBL (32), incubation of A3.01 cells with GM3 was followed by a significant decrease of CD4 staining on cell surface (Fig.1D), as compared with untreated cells (Fig. 1C). Together, this indicates that the cytoplasmic domain of CD4 plays a key role in the GM3-induced CD4 endocytosis. In order to verify a possible PKC involvement in the GM3-induced CD4 down-modulation, we performed cytofluorimetric analysis of the CD4 surface expression in the presence of different PKC inhibitors. We analyzed the GM3 effect in the presence of bisindolylmaleimide GF-109203X, a highly selective PKC inhibitor (27), or rottlerin, which inhibits selectively the Ca^{2+} independent isozyme PKC-δ (28). The results showed that in the presence of bisindolylmaleimide GF109203X (Fig.1E) or rottlerin (Fig. 1F), the GM3-induced CD4 down-modulation was inhibited. This finding suggests that the GM3 specific effect is PKC-dependent. Moreover, the inhibition by rottlerin is indicative of a PKC-δ involvement, confirming our previous observations on human PBL (22).

PKC-δ translocation following GM3 treatment occurs in both A2.01/CD4-cyt399 and A3.01

We then investigated the PKC-δ translocation from the cytosol to the plasma membrane in GM3-treated A2.01/CD4-cyt399 and A3.01 cells. This change of localization pattern is generally associated with enzyme activation (33). As shown in **Fig. 2**, after 5 min of treatment with GM3, translocation of PKC-δ was evident in A2.01/CD4-cyt399 (Fig. 2B), as well as in A3.01 cells (Fig. 2D), as revealed by scanning confocal microscopy. In GM3-treated cells, the anti-PKC-δ signal appeared uneven and punctated over the plasma membrane, whereas in untreated cells PKC isozymes were mostly diffuse in the cytoplasm (Fig. 2A, 2C). The clustered distribution of the PKC-δ suggests that the enzyme translocates mostly in correspondence of specific membrane domains.

These findings indicate that PKC activation by GM3 is upstream of the CD4 cytoplasmic domain phosphorylation.

cPLA2 activation following GM3 treatment occurs in both A2.01/CD4-cyt399 and A3.01

To an attempt to characterize the early biochemical pathway(s) induced by GM3 treatment, we carried out a series of experiments to assess the activity of cytosolic in A2.01/CD4-cyt399 and A3.01 cells. To assess the possibility of GM3 inducing $cPLA_2$ activation, extracts from GM3 treated or untreated cells were assayed for PLA_2 activity by analyzing AA release from radiolabeled AA-PC vesicles. In both cell types, the time course of $cPLA_2$ activity (**Fig. 3**) revealed a rapid AA release from PC vesicles that was maximal at 1–2 min and declined thereafter. The observed AA generation could be attributed to $cPLA_2$ activity, since AACOCF3, a specific inhibitor of $cPLA_2$ (34), totally inhibited PC hydrolysis (not shown).

These findings indicate that $cPLA_2$ activation by GM3 is upstream of CD4 phosphorylation in the cytoplasmic tail of the molecule.

ERK activation following GM3 treatment

Several studies have shown a role for ERKs in the phosphorylation and activation of $cPLA_2$ (35, 36). To investigate whether activation of ERKs might also be an early event following GM3 treatment, serum-starved, subconfluent A2.01/CD4-cyt399 and A3.01 were treated with 50 μg/ml GM3, and then cell-free lysates were probed with anti-phospho-p44/42 MAP kinase. The results clearly indi-

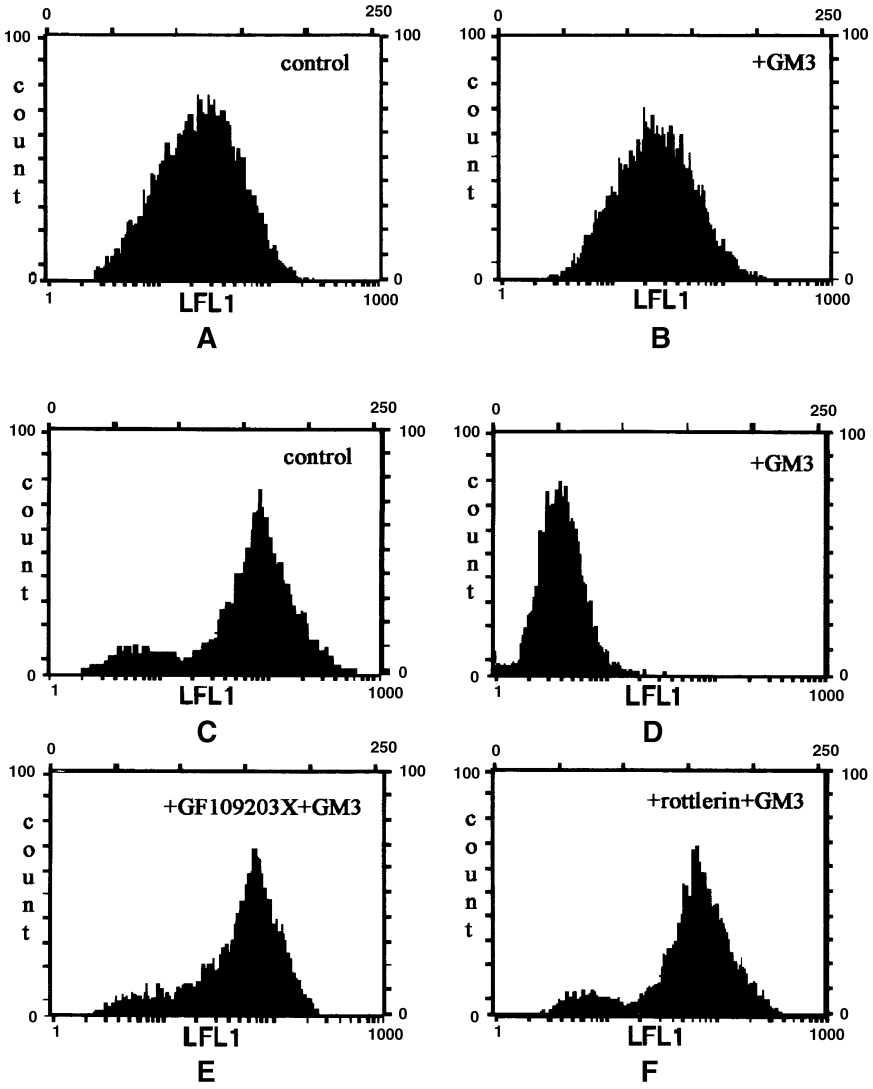

Fig. 1. GM3-induced CD4 down-modulation in the presence of protein kinase C (PKC) inhibitors. Cells (1×10^6 in 1 ml of PBS) were incubated in the presence of the following PKC inhibitors: bisindolylmaleimide GF109203X 50 μM, or rottlerin 10 μM. After a 15 min incubation time, GM3, 50 μg/ml, was added for 30 min at 37°C. Untreated and treated cells were stained directly using fluorescein-conjugated anti-CD4 MAb and analyzed by flow cytometry. A: A2.01/CD4-cyt399 cells incubated in the absence of GM3; B: A2.01/CD4-cyt399 cells incubated in the presence of GM3. C: A3.01 cells incubated in the absence of GM3; D: A3.01 cells incubated in the presence of GM3; E: A3.01 cells incubated in the presence of bisindolylmaleimide GF109203X, and then with GM3; F: A3.01 cells incubated in the presence of rottlerin, and then with GM3; Histograms represent log fluorescence versus cell number, gated on lymphocyte population of a side scatter/forward scatter (SS/FS) histogram. Cell number is indicated on the y-axis and fluorescence intensity is represented in three logarithmic units at the x-axis.

cated that in both cell lines GM3 induced ERK phosphorylation. **Figure 4A** shows the GM3 time-dependent effect in A3.01 cells was evident after as little as 1 min of incubation with GM3 and increased after 2–5 min (about 6-fold above basal levels) (Fig.4B). Similar findings were found in A2.01/CD4-cyt399 cells (data not shown).

To determine whether ERK phosphorylation in lymphocytes could also be stimulated by GM3, human PBL were treated with 50 μg/ml GM3, and cell-free lysates were then probed with anti-phospho-p44/42 MAP kinase. Again, the results (**Fig. 5A**) showed that GM3 induced ERK phosphorylation. In order to better investigate this activation, cell-free lysates were Western blotted onto nitrocellulose and probed with polyclonal anti-ERKs, which are reactive with both ERK-1 and ERK-2. Our results revealed the presence of 44 and 42 kDa bands, which reacted with the antibody either in the presence or in the absence of GM3 (Fig. 5B). After stripping of the membrane, the 42 kDa band reacted with the monoclonal anti-phosphotyrosine antibody only in GM3-treated cells, indicating that ERK-2 was tyrosine phosphorylated following GM3 treatment (Fig. 5B). This finding was confirmed by a different

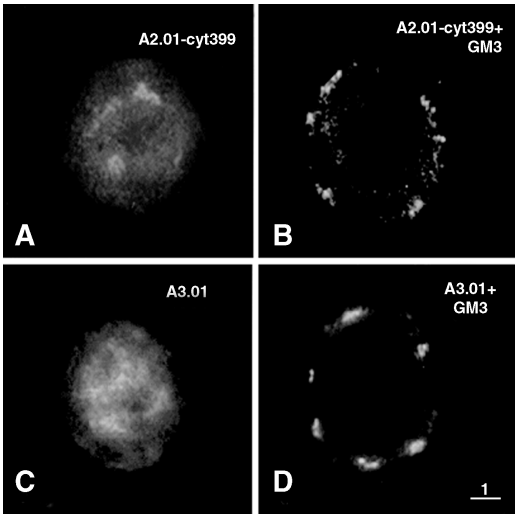

Fig. 2. PKC redistribution after GM3 treatment. A2.01/CD4-cyt399 and A3.01 cells (1×10^6 in 1 ml of PBS), were incubated with GM3, 50 μg/ml, for 5 min at 37°C. GM3 treated and untreated cells were then fixed with acetone-methanol 1:1 (v/v) for 10 min at 4°C. Cells were labeled with anti-PKC-δ for 1h at 4°C. After three washes in PBS, cells were incubated with FITC-conjugated goat anti-mouse IgG for 45 min at 4°C. The redistribution of PKC-δ was analyzed by scanning confocal microscopy. Lane A: untreated A2.01/CD4-cyt399 cells; lane B: GM3-treated A2.01/CD4-cyt399; lane C: untreated A3.01 cells; lane D: GM3-treated A3.01 cells. Scale bar: 1 μm.

approach. Human PBLs were incubated for 5 min in the presence or in the absence of GM3 and then lysed as reported above and immunoprecipitated with the polyclonal anti-ERKs antibody. Western blot analysis of these immunoprecipitates, performed using anti-phosphotyrosine antibody, demonstrated that GM3 exposure induced a significant ERK-2 phosphorylation as early as 5 min after treatment (Fig. 5C). This GM3-induced phosphorylation was completely abolished by previous incubation (30 min) with the synthetic MEK inhibitor PD98059, which is known to specifically prevent MEK-1 activation without affecting the activity of other kinases (29).

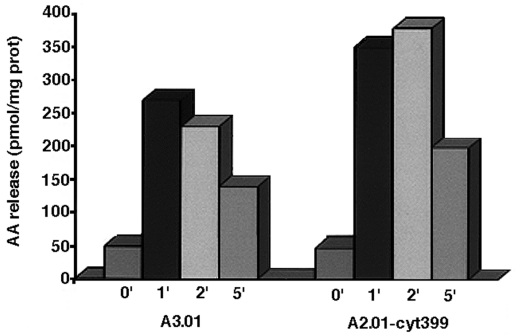

Fig. 3. Cytosolic phospholipase A_2 ($cPLA_2$) activation following GM3 treatment. A3.01 and A2.01/CD4-cyt399 cells (10^6/ml) were treated with GM3 (50 μg/ml) for the indicated times. $cPLA_2$ activity in cell lysates was tested against radiolabeled arachidonyl-PC vesicles. AA generation was analyzed by TLC and quantitated. Results are expressed as AA release and are representative of one from three independent experiments. Mean values of two determinations are reported. SD <5% of mean values.

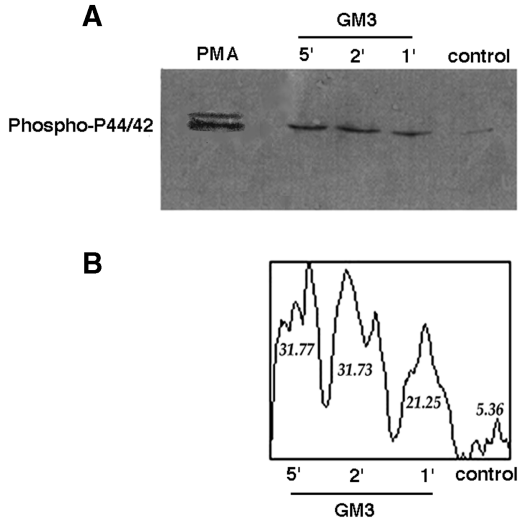

Fig. 4. MAPK p44/42 phosphorylation induced by GM3 in A3.01 cells. A: Cells were treated for the indicated times (1, 2, 5 min) with GM3 (50 μg/10^6 cells/ml) or, as a control, with PMA (50 ng/ml). The pellets of cell lysates, resuspended in loading buffer, were resolved on 12% SDS-PAGE under reducing conditions. The reactivity with monoclonal anti-phospho-p44/42 mitogen-activated protein kinase (MAPK) was analyzed by immunoblotting. Bound antibodies were visualized with HRP-conjugated anti-mouse and immunoreactivity assessed by chemiluminescence. B: Densitometric scanning analysis of the immunoblotting with anti-phospho-p44/42 MAP kinase MAb. Arbitrary units.

DISCUSSION

Several studies have tested the ability of specific gangliosides to interact with and affect the cell surface expression and activities of specific plasma membrane receptors. Previously, we showed that exogenously added GM3 to human peripheral T lymphocytes induced $cPLA_2$ activation, arachidonic acid release, PKC activation, CD4 serine phosphorylation, and dissociation from the CD4-linked $p56^{lck}$, resulting in CD4 endocytosis (22). The present study further elucidated the GM3 effect on the signaling pathway leading to CD4 endocytosis.

With this aim, we preliminarily used two different derivatives of the CEM T-cell line, one transfected with a mutant form of CD4 lacking the bulk of the cytoplasmic domain (A2.01/CD4-cyt399) and one expressing wild-type CD4 (A3.01) (25). Cytofluorimetric analysis of both T-cell lines, performed using an anti-CD4 antibody that recognizes the juxtamembrane epitope of the molecule, revealed that only A3.01 cells expressing wild-type CD4 showed a GM3-induced CD4 down-modulation. Since it did not occur in the cells lacking the bulk of the cytoplasmic domain, these new findings strongly suggest that this portion of the molecule, including the most efficient serine phosphorylated sites (Ser408, Ser415, Ser431) (31), plays a key role in the GM3-triggered CD4 endocytosis, as well as in phorbol ester-induced CD4 endocytosis (37). Thus, we suggest that serine phosphorylation is a required event for GM3-induced CD4 endocytosis.

In our previous paper (22), we hypothesized that in human T lymphocytes exogenous GM3 induced $cPLA_2$ acti-

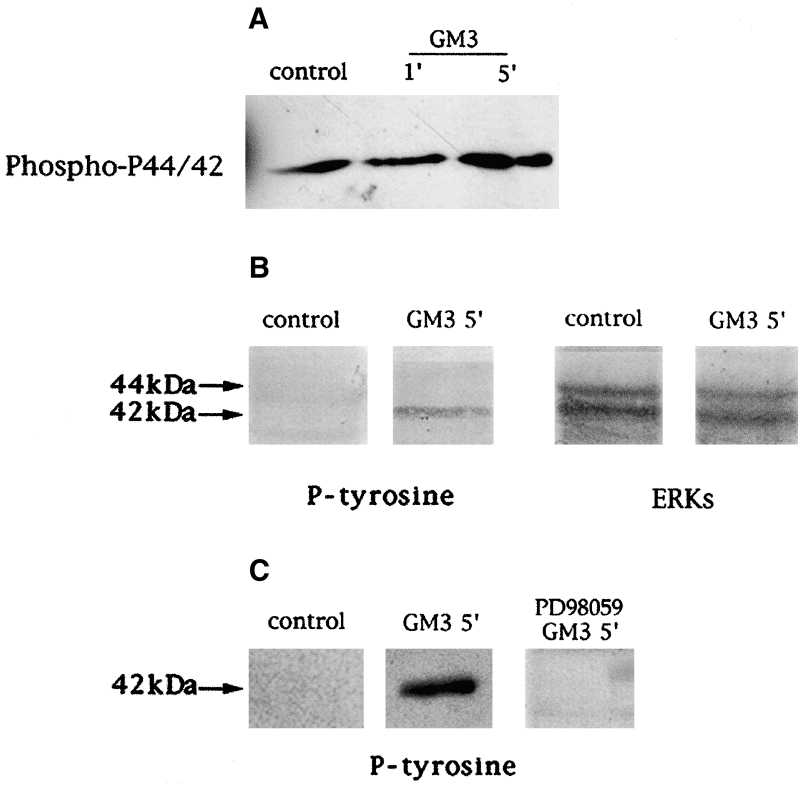

Fig. 5. Extracellular signal-regulated kinase (ERK) phosphorylation induced by GM3 in human peripheral blood lymphocytes (PBL). A: Cells were treated for the indicated times (1, 5 min) with GM3 (50 μg/10^6 cells/ml). The pellets of cell lysates, resuspended in loading buffer, were resolved on 12% SDS-PAGE under reducing conditions. The reactivity with monoclonal anti-phospho-p44/42 MAPK was analyzed by immunoblotting. Bound antibodies were visualized with HRP-conjugated anti-mouse and immunoreactivity assessed by chemiluminescence. B: Untreated and GM3-treated cells (5 min, 50 μg/10^6 cells/ml) were lysates. The pellets resuspended in loading buffer were resolved on 12% SDS-PAGE under reducing conditions. The reactivity with polyclonal anti-ERKs was analyzed by immunoblotting. Bound antibodies were visualized with HRP-conjugated anti-rabbit and immunoreactivity assessed by chemiluminescence. Manufacturer-specified protocols were used to strip the membrane to reprobe with anti-phosphotyrosine MAb and then with HRP-conjugated anti-mouse IgG. C: Cell-free lysates from untreated, GM3-treated cells (5 min, 50 μg/10^6 cells/ml) and GM3-treated cells (5 min, 50μg/10^6 cells/ml), previously incubated with 50 μM MEK inhibitor PD98059 (30 min), were immunoprecipitated with polyclonal anti-ERKs and then with protein G-sepharose beads. The mixtures were centrifuged and washed three times with 0.4 ml of the RIPA buffer. The pellets resuspended in loading buffer were resolved on 12% SDS-PAGE under reducing conditions and immunoreactivity with anti-phosphotyrosine MAb was assessed as above.

vation with arachidonic acid release, which in turn led to PKC-δ translocation, CD4 phosphorylation, CD4-$p56^{lck}$ dissociation, and receptor endocytosis. Thus, we analyzed the GM3-triggered signaling pathway in the two lymphoblastoid cell lines (A2.01/CD4-cyt399 and A3.01). Interestingly, although CD4 endocytosis occurred only in wild-type CD4 cells, GM3 was able to induce $cPLA_2$ activation, with consequent arachidonic acid release and PKC-δ translocation in both cell lines. Thus, our findings on T-cell lines demonstrate that the biochemical pathway triggered by GM3 is not consequent to CD4 endocytosis, suggesting that the GM3-triggered events precede the CD4 down-modulation.

Moreover, it was of interest to analyze the molecular signals triggering GM3-induced $cPLA_2$ activation. Our findings indicated that GM3 treatment leads to rapid ERK phosphorylation in lymphoblastoid T-cells, as well as in human lymphocytes. This effect was specific for ERK-2, as revealed by anti-phosphotyrosine binding on both cell-free lysates and ERK immunoprecipitates. Although a direct stimulation of ERK by GM3 is unlikely, the observation that cell incubation with the MEK-1 inhibitor PD98059 prevented ERK phosphorylation, as well as CD4 endocytosis (22), strongly suggests that GM3 stimulates a signaling cascade involving MEK-1 or a MAP kinase-related protein that subsequently activates ERK.

In human lymphocytes, GM3 is present in relatively detergent-resistant microdomains of the plasma membrane, where some transducer proteins are highly enriched (5). These plasma membrane microdomains of distinct proteins (Src family, tyrosine kinases, G proteins, PKC, and CD4) and lipids (GM3, sphingomyelin, cholesterol) correspond to the glycosphingolipid-enriched microdomains (GEM) (4, 38), which are involved in lymphocytic func-

tions such as signal transduction, cell activation, and/or endocytic process of specific membrane antigens. Indeed, they may function as binding factors for many different molecules, including complementary glycosphingolipids, antibodies, and selectins, and are involved in transducing stimulatory and/or inhibitory cellular signals (39). Among these, the present study provides evidence that GM3 is active in the mitogenic signal transduction pathway.

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