Cell Growth Regulation through GM3-enriched Microdomain (Glycosynapse) in Human Lung Embryonal Fibroblast WI38 and Its Oncogenic Transformant VA13*

Marcos S. Toledo†, Erika Suzuki‡, Kazuko Handa‡, and Senitoh Hakomori§∥

From the †Pacific Northwest Research Institute, Seattle, Washington 98122-4302 and the Departments of §Pathobiology and ¶Microbiology, University of Washington, Seattle, Washington 98195

Cell growth control mechanisms were studied based on organization of components in glycosphingolipid-enriched microdomain (GEM) in WI38 cells versus their oncogenic transformant VA13 cells. Levels of fibroblast growth factor receptor (FGFR) and cSrc were 4 times and 2–3 times higher, respectively, in VA13 than in WI38 GEM, whereas the level of tetrapsanin CD9/CD81 was 3–5 times higher in WI38 than in VA13 GEM. Csk, the physiological inhibitor of cSrc, was present in WI38 but not in VA13 GEM. Functional association of GEM components in control of cell growth in WI38 is indicated by several lines of evidence. (i) Confluent, growth-inhibited WI38 showed a lower degree of FGF-induced MAPK activation than actively growing cells in sparse culture. Both processes i and ii were inhibited by GM3 since they were enhanced by GM3 depletion with n-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol (P4). (iii) The high level of inactive cSrc associated with growth-inhibited cells was caused by co-existing Csk in WI38 GEM. (iv) Interaction of GM3 with FGFR was demonstrated by binding of GM3 to FGFR in the GEM fraction, as proved with GM3-coated beads, and by confocal microscopy. In contrast to WI38, both cSrc and MAPK in VA13 were strongly activated regardless of FGF stimulation or GM3 depletion by P4. Continuous, constitutive activation of both cSrc and MAPK was due to (i) a much higher level of cSrc and FGFR in VA13 than in WI38 GEM, (ii) their close association/interaction in VA13 GEM as indicated by clear co-immunoprecipitation between cSrc and FGFR, and (iii) the absence of Csk in VA13 GEM, making GEM incapable of inhibiting cSrc activation.

Cell proliferation and motility are inhibited when non-transformed cells come into contact in culture; this phenomenon was termed “contact inhibition” by Abercrombie and colleagues ~50 years ago (1). In contrast, transformed cells do not change their growth behavior or motility regardless of cell population density (“loss of contact inhibition”) (2). Cell surface sialic acid and sialo-glycoconjugates were considered to be involved in contact inhibition (3). Cell surface gangliosides and their changes associated with oncogenic transformation were suggested during the early 1970s to be closely associated with loss of contact inhibition (4, 5), and this concept has recently been confirmed (6). However, the mechanism for the functional role of gangliosides in defining contact inhibitiability and cell growth remains unclear. The aim of the present study was to explore this mechanism with focus on interaction of gangliosides with growth factor receptors and signal transducers in membrane microdomains.

Some non-transformed epithelial or fibroblast cell lines (e.g. BSC-1, baby hamster kidney, and NIH) do not display clear contact inhibition of cell growth; the apparent reduction of cell growth at high cell population density is often caused by exhaustion of nutrient or growth factor in medium rather than by cell contact (7, 8). On the other hand, other types of cell lines, such as endothelial cells and corneal epithelial cells, show clear contact inhibition regardless of nutrient or growth factor level in medium or other growth conditions (9). The human diploid cell line WI38, derived from normal embryonal lung (10, 11), displays clear contact inhibition of cell growth, whereas its SV40 virus transformant VA13 (12) is characterized by loss of contact inhibition. Since WI38 is diploid (and may show phenotypic variability depending on number of passages), whereas VA13 is haploid (and its phenotype is invariant), comparison of growth control mechanism in these two types of cells should be made using defined, early stage passage of WI38. Recently criteria for loss of growth control mechanisms were studied using VA13 as compared with WI38 (13).

Many studies during the past decade have focused on regulation of cell growth, adhesion, and motility through membrane microdomains, particularly the glycosphingolipid-enriched microdomain (GEM) (14, 15) in which glycosphingolipids (GSLs) are associated with signal transducers (Src family kinases and small G-proteins) (Refs. 14–21; for reviews, see Refs. 22 and 23). Microdomains in B16 melanoma cells display GM3-dependent adhesion to Gg3-coated plates, leading to activation of associated cSrc and RhoA (15, 17) and enhanced motility (24).

The abbreviations used are: GEM, glycosphingolipid-enriched microdomain; β-CD, β-cyclodextrin; C/M, chloroform/methanol; co-IP, co-immunoprecipitation; HPTLC, high performance thin-layer chromatography; HSFG, heparan sulfate proteoglycan; IP, immunoprecipitation; LacCer, lactosylceramide; MAPK, mitogen-activated protein kinase; P4, n-threo-1-phenyl-2-palmitoylamino-3-pyrrolidino-1-propanol; PBS, phosphate-buffered saline; SFG, sialylgalactobioside (sialyl-lacto-neotetraosylceramide); SV, sodium vanadate; GM1, Galβ1,3GalNAcβ1,4Galβ1,1Cer; GM2, GalNAcβ1,4Galβ1,3Galβ1,1Cer; GM3, NeuAcα2,3Galβ1,4Glcβ1,1Cer; GSL, glycosphingolipid; Gg3, GaINAcβ1,4Galβ1,4Glcβ1,1Cer; P4, phospho-Aβ; mAb, monoclonal antibody; HRP, horseradish peroxidase; FITC, fluorescein isothiocyanate; Fr.; fraction(s); MØ, serum-free medium; SH, Src homology; Csk, C-terminal Src kinase; FGFR, fibroblast growth factor; FGF, fibroblast growth factor receptor; GB4, GaINAcβ1,3Galβ1,4Galβ1,4Glcβ1,1Cer.

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†To whom correspondence should be addressed: Pacific Northwest Research Inst., 720 Broadway, Seattle, WA 98122-4302. E-mail: hakomori@uwashington.edu.

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The GM3-enriched microdomain was immunoseparated from cholesterol/caveolin-enriched "caveolar membrane" (18). On the other hand, GM3-enriched microdomains in colorectal cancer (25) and bladder cancer (26) were also enriched in tetraspanin CD9, and tumor cell motility and invasiveness appeared to be inhibited by coexpression of GM3 and CD9. Model experiments using Chinese hamster ovary cell mutant IdlD transfected with tetraspanin CD9 or CD82 showed that integrin-tetraspanin-GM3 complex inhibited cell motility/invasiveness (27, 28). In these previous studies, however, regulation of tumor cell phenotype through microdomain components could not be compared with that of non-transformed progenitor cells since progenitors of melanoma, colorectal, and bladder cancer cells were not available. We therefore studied contrasting composition and functional properties of GEM of WI38 versus VA13 cells. We hereby report that GEM components, their organization, and their interaction provide the basis for growth control in WI38 and loss of growth control in VA13.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents

Human embryonic lung fibroblast cell line WI38 (10, 11) and SV40-transformed WI38, termed VA13 (12), were from American Type Culture Collection (ATCC, Manassas, VA). WI38 cells with passage numbers 18–20 were used. They were grown in minimum essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in 5% CO2.

Antibodies—Anti-P-MAPK (p42/44) rabbit IgG, anti-MAPK (p42/44) rabbit IgG, anti-P-Src (Tyr-416) rabbit IgG, anti-P-Src (Tyr-527) rabbit IgG, anti-P-Lyn (Tyr-507) rabbit IgG, and anti-P-Phos (Tyr-653/684) rabbit IgG (Cell Signaling Technologies, Beverly, MA); anti-c-Src (SRC2) rabbit IgG, anti-caveolin-1 rabbit IgG, anti-Lyn rabbit IgG, anti-c-Csk rabbit IgG, and rabbit IgG "FGFR-3" directed to the cytoplasmic domain of FGFR-3 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); mouse IgG1 mAb "FGFR(AB1)" directed to FGFR-1, -2, -3, and -4 (Oncogene Science, Cambridge, MA); anti-Cd81 mouse IgG2a (Beckman Coulter, Inc., Brea, CA); anti-Cd9 mouse IgG1 (BD Biosciences); anti-β-actin mouse IgG (Sigma); and mouse mAb directed to heparan sulfate proteoglycan (Chemicon International, Temecula, CA) were used.

Secondary Antibodies—Goat anti-rabbit IgG-HRP (Transduction Laboratories, Lexington, KY) and goat anti-mouse Ig-HRP (Santa Cruz Biotechnology, Inc.) were used.

Fluorescence-conjugated Antibodies—Anti-mouse IgG1-FITC, anti-mouse IgG2a-FITC, anti-mouse IgG3-FITC, and anti-rabbit IgG-FITC (Santa Cruz Biotechnology, Inc.) and anti-mouse IgG1-Alexa Fluor 594 and anti-mouse IgG3-Alexa Fluor 594 (Molecular Probes, Eugene, OR) were used.

Other Reagents—The Micro-BCA kit (Pierce, and acidic FGF-1 and basic FGF-2 (recombinant products) were used.

GEL Preparation and Determination of GEM Components

GEM, as originally defined (14, 15), was prepared as described previously (17, 18). Briefly, -4 x 106 cells, prepared as above, were washed with cold PBS (8.1 mM Na2HPO4, 1.5 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, pH 7.4) containing 5 mM sodium vanadate (SV), harvested by rubber scraper, and washed twice with the buffer. The pellet was resuspended in 1.2 ml of ice-cold Brij98 lysis buffer containing 1% Brij98, 25 mM Hepes buffer, pH 7.5 (ICN Biomedicals Inc.) plus 75 units/ml aprotinin, 2 mM phenylmethanesulfonyl fluoride, and 5 mM SV. In a separate experiment, the cell pellet was lysed in the same buffer but containing 0.5% or 1% Triton X-100 instead of 1% Brij98. In either case, the suspension was kept at 4 °C for 30 min and Dounce-homogenized (-15 strokes). The lysate was centrifuged at 2000 rpm for 5 min to remove the nucleus and cell debris. The postnuclear supernatant fraction was subjected to sucrose density gradient centrifugation to separate the low density membrane fraction as described previously (18). Twelve fractions, 1 ml each, were collected from top to bottom and termed "Fr. 1–12." The protein content of each fraction was determined using the Micro-BCA kit. Aliquots of each fraction containing equal protein content (~1 µg) were analyzed by SDS-PAGE and Western blot. In some experiments, the intensity of the Western blot was determined by densitometry using the Scion imaging program.

Effect of Pretreatment of Cells with β-CD or Saponin on GEM Composition

WI38 and VA13 cells at 90–95% confluence were incubated in serum-free medium for 12 h, harvested by rubber scraper, and centrifuged. The cell pellet was treated with 10 mM β-CD for 1 h at room temperature or 0.2% saponin for 10 min at 4 °C under gentle rotation, washed three times with ice-cold PBS plus SV, suspended, lysed with 1% Brij98 lysis buffer, and subjected to sucrose density gradient centrifugation as described in the preceding section. Each fraction was analyzed by Western blot as described above. When cell monolayers instead of cell pellets were treated directly with β-CD or saponin, effects were not clear.

GM3 Composition of Various Fractions

WI38 and VA13 cells were grown to ~90% confluence in 150-mm dishes, harvested, and washed three times with PBS. GSLs were extracted with 10 volumes of isopropanol/hexane/water (55:25:20, v/v/v) and once with C/M (2:1, v/v). Extracts were evaporated, and GSLs were dissolved in C/M (2:1, v/v), analyzed by HPTLC and stained with orcinol/sulfuric acid staining (1 g of orcinol in 50 ml of sulfuric acid and 450 ml of H2O) or mouse anti-GM3 mAb DH2 (IgG3).

In another experiment, cells were treated with P4 as described below and then processed. For HPTLC, the orcinol/sulfuric acid staining and HPTLC-immunostaining, the protein content of the original cell suspension was determined, GSL fractions were prepared, and aliquots of GSL fractions equivalent to ~20 µg of cellular protein were loaded in each spot.
FGFR interaction with cSrc in VA13 GEM was determined by co-IP using the ProFound™ mammalian co-IP kit (Pierce) according to the manufacturer’s instructions. Briefly 100 µg of anti-FGFR antibody was immobilized with 100 µl of antibody coupling gel. The GEM fraction (containing ~100 µg of protein) was incubated (at 4 °C overnight) with anti-FGFR-affixed gel, washed three times with co-IP buffer, and eluted.
four times. Each eluate was subjected to SDS-PAGE with Western blot analysis to determine components that interact with FGFR. Normal mouse IgG instead of anti-FGFR was used as control.

**Interaction of FGFR in GEM with GM3 and Other GSLs Using Polyostyrene Beads Coated with GSL**

10 μg of GM3, GM2, GM1, SPG, GB4, or LacCer was dried in a glass tube, dissolved in ethanol/water (9:1), mixed with 2.5 × 10^5 polyostyrene beads coated with GSL (Interfacial Dynamics Corp., Portland, OR; previously washed in PBS and suspended in ethanol), dried under a stream of N2, and washed three times with PBS. Beads were incubated with 100 μl of 0.1% gelatin for 1 h at room temperature to block nonspecific binding sites, the suspension of beads in gelatin was diluted 10-fold with PBS and centrifuged, and beads were suspended in 300 μl of PBS containing 20 μg of protein of Fr. 5 (GEM fraction) from WI38. The mixture was kept for 2 h at 4 °C under rotary agitation and then washed three times with PBS, protein bound to GM3-coated beads was solubilized with 50 μl of SDS-PAGE sample buffer, and 20-μl aliquots were subjected to SDS-PAGE with Western blotting with anti-FGFR.

To observe the effect of sialic acid on FGFR binding, GM3-coated beads were treated with 0.1 unit of Chlostridium perfringens sialidase for 1 h at 37 °C and washed with PBS prior to incubation with the GEM fraction.

**Analysis by Confocal Microscopy**

Interactions between GEM components were analyzed as described previously (28). Briefly, WI38 or VA13 cells (3 × 10^4) were grown on a cover glass (diameter, 18 mm) placed in a 6-well plate for 24 h. The cover glass with cells was removed, washed three times with ice-cold PBS, and fixed with 3.7% paraformaldehyde, PBS for 15 min at 4 °C. Fixed cells were washed three times with PBS and incubated with 1% bovine serum albumin, 0.1% NaN3, PBS (buffer A) for 30 min. For double staining, cells were incubated with 100 μl of antibody mixture diluted 200-fold in buffer A for 1 h; antibodies used were anti-CDC42 (mouse IgG1, IgG3), anti-CDC42, and anti-MM3 DH (mouse IgG3), anti-FGFR (Ab-1) (mouse IgG1), and anti-FGFR-3 (rabbit IgG). For secondary antibody mixtures, FITC-conjugated antibodies were diluted 20-fold, and Alexa Fluor-conjugated antibodies were diluted 50-fold; antibodies used were anti-mouse IgG1-Alexa Fluor, anti-mouse IgG1-Alexa Fluor, anti-mouse IgG1-Alexa Fluor, and anti-rabbit IgG-FITC diluted in buffer A for 1 h. After each step, cells were washed with buffer A. Stained cells were mounted with a drop of Glycergel mounting medium (Dako Corp., Carpiniteria, CA) containing 1% 1,4-diazabicyclo[2.2.2]octane (fluorescence stabilizer). Fluorescence was observed by laser scanning confocal microscopy (Leica Model TCS-SP) at excitation wavelengths of 594 nm and 488 nm and emission wavelengths of 546 nm and 520 nm, respectively. The relative fluorescence of each component was quantified using Leica ASPL (Leica Imaging Systems Ltd., Bannockburn, Illinois)

**Effect of GM3 Depletion by P4 on WI38 Cell Proliferation**

WI38 cells (2 × 10^5/well) were seeded in 48-well plates and incubated in culture medium. After 8 h medium was changed to culture medium alone or culture medium + 1 μm P4. After 48 h cells were starved by incubation for 24 h in serum-free medium and then incubated overnight with or without 1 μM P4. Next cells were incubated with anti-M0, M0 + 10 ng/ml FGF, M0 + 10 ng/ml FGF + anti-mouse IgG instead of anti-FGFR was used as control.

**Effect of Sulfation Inhibitor (Sodium Chlorate, NaClO3) on GM3 Level in GEM of WI38 and VA13 Cells**

**Effect of Sulfation Inhibitor (Sodium Chlorate, NaClO3) on FGF-dependent MAPK Activation**

**Relative Quantity of Components in GEM from WI38 Versus VA13 Cells**

**Results**

Contact Inhibitability of Cell Growth in WI38 and Its Absence in SV40-transformed VA13 Cells—A striking difference in growth behavior of WI38 versus VA13 cells is shown in Fig. 1. WI38 cells reached saturation density (1.7 × 10^5/cm^2) by day 8–9, whereas VA13 cells were still in logarithmic growth phase at day 8 with cell population density < 2 × 10^5/cm^2.

**Relative Quantity of Components in GEM from WI38 Versus VA13 Cells—GEM components, prepared in 1% Brij98 or 1% Triton X-100 lysis buffer, are shown in Fig. 2A. GM3 (essentially the sole GSL component in both cell lines) was concentrated in GEM (Fr. 4–6) and absent in high density, soluble fractions (Fr. 10–12) (Fig. 2A-1). Six protein components (FGFR, cSrc, Lyn, tetratraspanins CD9 and CD81, and caveolin) were detected in both WI38 and VA13 GEM prepared in 1% Brij98 lysis buffer. Of these, FGFR, CD9, and CD81 were solubilized in both 0.5% (not shown) and 1% Triton X-100, whereas GM3, cSrc, Lyn, and caveolin were resistant to solubilization and stayed in the GEM fraction (Fig. 2, A-2). FGFR in WI38 and VA13 was identified as FGFR type 3 because it reacted with antibody specific for FGFR type 3 (Western blot, data not shown).

**Quantities of FGFR, cSrc, CD9, and CD81 present in the GEM (Fr. 5) and soluble (Fr. 12) fractions prepared in 1% Brij98 lysis buffer were analyzed with SDS-PAGE and Western blotting (Fig. 2B).** Further quantitative densitometry of each band from GEM using the Scion imaging program is shown in Fig. 2C. Remarkably FGFR in GEM was 4–5 times higher in VA13 than in WI38, and cSrc in GEM was 2–3 times higher in VA13 than in WI38. In contrast, CD9 and CD81 in GEM were 3–4 times higher in WI38 than in VA13. The differences in levels of these components between WI38 and VA13 GEM were unchanged regardless of sparse versus confluent growing conditions. Levels of caveolin and GM3 were not significantly different between WI38 and VA13 (see “GM3 Level in GEM of WI38 and VA13 Cells and Its Depletion by P4 Treatment”.)
below). Levels of G-proteins such as RhoA and Ras in both WI38 and VA13 were very low (data not shown).

Comparative Resistance of Components in GEM from WI38 Versus VA13 Cells to Saponin Treatment—Components associated with cholesterol in microdomain can be reduced or eliminated from the domain by treatment with cholesterol-binding reagents β-CD, filipin, or nystatin (18, 29–31). In the current study, however, treatment with 10 mM β-CD for 1 h at room temperature did not reduce the level of FGFR or cSrc in VA13 GEM but did reduce FGFR and cSrc in WI38 GEM. CD9 and CD81 in both WI38 and VA13 GEM were reduced to some extent, but the level of caveolin was unchanged (completely resistant). The Csk level in WI38 GEM was reduced (Fig. 3 and Table I).

Resistance of GEM components following pretreatment of cells with 0.2% saponin for 20 min indicates a degree of interaction of each component (32). We applied this condition to both WI38 and VA13. cSrc, Csk, and CD9 in WI38 GEM were reduced, whereas CD81 and caveolin in both WI38 and VA13 GEM were completely resistant to saponin pretreatment (Fig. 3 and Table I). Relative resistance of these components, including caveolin, to β-CD and saponin is in contrast with components of caveolar/raft membrane, which are known to be highly susceptible to β-CD and other cholesterol-binding reagents (see “Discussion”).

GM3 Level in GEM of WI38 and VA13 Cells and Its Depletion by P4 Treatment—Essentially the same level of GM3 was found from WI38 and VA13 cells by HPTLC analysis of C/M extract as detected by orcinol/sulfuric acid reagent and by immunostaining with GM3-specific mAb DH2 (Fig. 4A). The GM3 band from WI38 cells stained with DH2 included a minor slow migrating band that was absent in WI38. This minor band presumably contained hydroxy fatty acid (see “Discussion”). GM3 synthesis was blocked, and GM3 was completely depleted when WI38 and VA13 cells were treated with 1 μM P4 for 72 h (Fig. 4B). The inhibitory effect of 1 μM P4 on GM3 synthesis was similar for sparse growing versus confluent cells (Fig. 4B). Flow cytometric analysis using DH2 confirmed the effect of P4 on GM3 expression at the cell surface (data not shown).

Differential FGF- and GM3-dependent Activation Pattern of MAPK and cSrc in Confluent (Growth-inhibited) Versus Sparse (Actively Growing) WI38 Cells and Absence of Such Difference in VA13—The phosphorylation response of FGFR, cSrc, and MAPK following stimulation by 10 pg/ml acidic FGF of WI38 and VA13 cells, with or without GM3 depletion by P4, is summarized in Fig. 5A. Optimal MAPK activation, probed by antibody against phosphorylated p42/44, was found to be induced by 5 min of FGF stimulation (Fig. 5A, W, row 6). This activation response was stronger when GM3 was depleted (Fig. 5A, W, row 6, +P4). Under this condition, Tyr phosphorylation of FGFR (Fig. 5A, W, row 1) or of cSrc at Tyr-416 (Fig. 5A, W, row 3) was undetectable or minimally detectable.

FGFR phosphorylation may occur early (e.g., within 5 min) but disappear during the long assay procedure (see “Materials and Methods”). The same situation may apply to Src activation with Tyr-416 phosphorylation. In addition, failure of cSrc activation under this condition may result from enhanced phosphorylation at Tyr-527 caused by the presence of Csk in WI38 GEM (see “Association of Csk with GEM in WI38 Cells and Absence of Csk in VA13 GEM”).

Clear differences were observed in sparse versus confluent conditions.

**Table I**

| Quantity in GEM | Susceptibility to β-CD | Susceptibility to Saponin |
|-----------------|------------------------|--------------------------|
| GM3             | WI38 = VA13            | WI38 = VA13              |
| FGFR            | WI38 ≪ VA13            | WI38 ≪ VA13              |
| cSrc            | WI38 ≪ VA13            | WI38 ≪ VA13              |
| Csk             | Present only in WI38 GEM| NA                       |
| CD9             | WI38 ≫ VA13            | +                        |
| CD81            | WI38 ≫ VA13            | ±                        |
| Caveolin        | WI38 = VA13            | –                        |

- -, unchanged; +, reduced ~50%; ±, reduced ~10–20%; NA, not applicable because Csk is absent in VA13 GEM.

**FIG. 3.** Effect of β-CD and saponin on distribution pattern of GEM components in WI38 and VA13 cells. Nearly confluent cells were harvested and pretreated with β-CD or saponin as described under “Materials and Methods.” The effects of these treatments on distribution patterns of major components are shown by Western blotting pattern. Note that FGFR and cSrc were barely reduced in GEM from VA13 but were significantly reduced in GEM from WI38. Csk, CD9, and CD81 were slightly reduced in WI38, but caveolin was completely resistant to β-CD. All components in both WI38 and VA13 were resistant to saponin. P, postnuclear fraction.
Fig. 4. HPTLC immunostaining of total GSLs extracted from WI38 and VA13 cells. A, cells were grown, and GSLs were extracted and stained with mAb DH2 as described under “Materials and Methods.” B, cells were pretreated with (+) or without (−) P4, and GSLs were extracted and stained as in A. For both panels, GSLs extracted from ~20 μg of cell protein were loaded per lane. Note that the level of GM3 is essentially the same in WI38 as in VA13. GM3 was completely depleted by P4 treatment in both WI38 and VA13 regardless of sparse (S) versus confluent (C) condition. Similar results to those shown in A and B were obtained in three separate experiments.

Fig. 5. Src and MAPK status in response to FGF and depletion of GM3 in WI38 versus VA13 cells. A, sparse growing (S) and confluent (C) cultures of WI38 (W) and VA13 (V) cells were prepared as described under “Materials and Methods.” The stimulatory effect of 10 pg/ml acidic FGF and the inhibitory effect of GM3 (probed by P4 treatment) were determined as described under “Materials and Methods.” Three major points should be noted. (i) FGF-induced MAPK activation in sparse growing WI38 was inhibited by GM3 as probed by P4 treatment (W, S, row 6). This inhibitory effect was stronger in confluent cells (W, C, row 6). (ii) Inactive Src with Tyr-527 was higher in confluent (W, C, row 4) than in sparse growing (W, S, row 4) WI38. The inactive Src expression was inhibited by GM3 (in the absence of P4) (W, S, row 4, two right lanes). (iii) Both MAPK and Src activation in VA13 were observed regardless of cell population density or P4 treatment (V, S, rows 3, 4, and 6; V, C, rows 3, 4, and 6). B, [3H]thymidine incorporation of WI38 after P4 treatment and FGF stimulation. Cells were seeded and processed as described under “Materials and Methods.” The statistical difference for each of the comparisons indicated by a bracket (*, between P4-treated versus P4-non-treated cells; **, between FGF-treated versus FGF-non-treated cells) was significant at the p < 0.001 level.

Fig. 6. Presence of Csk in WI38 GEM fraction. GEM fractions from sparse and confluent WI38 and VA13 cultures were analyzed for the presence of the Src family inhibitor Csk. Cells were processed as described under “Materials and Methods” using 1% Brij98 lysis buffer. After protein quantification, 2.3 μg of protein was loaded on an 8% gel and analyzed by Western blot using anti-Csk Ab (Santa Cruz Biotechnology, Inc.). Note that Csk was present in WI38 GEM (Fr. 5 and 6) but absent in VA13 GEM. P, postnuclear fraction.

Confluent culture vs Sparse culture

Presence of Csk in GEM fraction

Cell Growth Regulation through Glycosynapse in WI38 and VA13

Discussion

VI38 culture. (i) MAPK phosphorylation was much lower in confluent than in sparse culture in both the presence and absence of GM3 (Fig. 5A, W, row 6, S versus C, +P4 versus -P4). Pretreatment with P4 enhanced MAPK phosphorylation to a greater degree in sparse than in confluent culture, although the biochemical level of MAPK was constant regardless of growth condition (Fig. 5A, W, row 7, S and C). (ii) Inactive cSrc, characterized by Tyr-527 phosphate, was clearly higher in confluent than in sparse culture (Fig. 5A, W, row 4, S versus C). This difference was not clearly correlated with the presence or absence of GM3 or with FGF stimulation. Yet unknown mechanisms that control Src inactivation versus activation associated with confluent versus sparse cell growth may exist (see “Discussion”).

In contrast to WI38, VA13 showed strong cSrc activation through Tyr-416 phosphorylation (Fig. 5A, V, row 3) and MAPK activation (Fig. 5A, V, row 6) regardless of FGF stimulation, GM3 depletion, or confluent versus sparse culture.

The above findings suggest that FGF-dependent MAPK activation is inhibited by GM3 in WI38. This possibility was substantiated by the fact that FGF-dependent mitogenesis, determined by thymidine incorporation, occurred in P4-treated, GM3-depleted WI38 cells but not in P4-untreated cells (Fig. 5B).

Association of Csk with GEM in WI38 Cells and Absence of Csk in VA13 GEM—Csk was detected in GEM of WI38 under both sparse growing and confluent conditions, whereas Csk was not detected or was minimally detected in GEM from VA13 cells regardless of sparse or confluent condition (Fig. 6). The mechanism underlying the different localization of Csk in WI38 versus VA13 remains to be clarified (see “Discussion”).
Co-localization of FGFR, GM3, and Tetraspanins Revealed by Confocal Microscopy—Possible interaction of GEM components was studied by confocal microscopy as described under “Materials and Methods.” We observed the following: (i) clear co-localization in WI38 of GM3 with FGFR, with FGFR(Ab1) (which reacts with the extracellular domain of FGFR-1, -2, -3, and -4) (Fig. 7A, row 1) and less clear co-localization with Ab FGFR-3 directed to the cytoplasmic domain of FGFR-3 (Fig. 7A, row 2), (ii) in VA13, co-localization of GM3 with FGFR was clear with FGFR(Ab1) (Fig. 7B, row 1) but was less clear with Ab FGFR-3 (Fig. 7B, row 2), (iii) clear interaction of CD9 with CD81 (Fig. 7, A and B, row 3), (iv) weak interaction between FGFR-3 and CD9 and between FGFR(Ab1) and CD81 in VA13 (data not shown), and (v) weak interaction between GM3 and CD9 and between CD81 and GM3 in both WI38 and VA13 (data not shown).

Interaction of GM3 with FGFR in GEM Fraction Studied Using GM3-coated Beads—The possible interaction of GM3 with FGFR in the GEM fraction was further supported by studies using GM3-coated polystyrene beads as described under “Materials and Methods.” FGFR was clearly detected as a component bound to GM3-coated beads in the GEM fraction from WI38, indicating interaction of GM3 with FGFR. Levels of FGFR adhered to GM2-, GM1-, or SPG-coated beads were significant but lower than levels of FGFR bound to GM3-coated beads. Levels of FGFR bound to beads coated withGb4 or LacCer were very low (similar to non-coated control beads). Beads coated with GM3 and subsequently treated with sialidase also showed very low FGFR similar to control beads (Fig. 8).

Interaction of FGFR with cSrc in VA13 GEM Indicated by Co-IP—FGFR interaction with cSrc was shown by co-IP experiments using the ProFoundTM co-IP kit as described under “Materials and Methods.” Both FGFR and cSrc were detected by Western blot analysis in eluates from anti-FGFR antibody-affixed gel on which VA13 GEM was loaded. In a control experiment with a normal mouse IgG-affixed column, neither was detected (Fig. 9). Such a co-IP pattern of FGFR and cSrc was not found in similar experiments using WI38 GEM (data not shown).

Absence of HSPG in WI38 GEM and Lack of Effect of Sulfation Inhibitor on FGF-dependent MAPK Activation—Many previous studies indicate that FGF induces signaling through a FGF/HSPG/FGFR complex (e.g. Refs. 33 and 34 and references therein). We therefore considered the possibility that HSPG is involved in FGF-dependent signaling through FGFR-3 in GEM of WI38 cells as described above. This possibility was ruled out based on (i) lack of reactivity of WI38 GEM with anti-HSPG antibody in dot-blot assay (data not shown) and (ii) lack of effect of sulfation inhibitor NaClO3 on FGF-dependent MAPK activation in both sparse growing and confluent WI38 (data not shown).

DISCUSSION

Cell growth inhibition occurring at increased cell population density is assumed to depend on cell contact and is classically termed “contact inhibition of cell growth.” In many oncogenically transformed cells, growth control is not restricted by population density; this response is termed loss of contact inhibition (1, 2). Enhanced synthesis of certain GSLs in response to cell growth arrest and loss of such a GSL response in transformed cells (4–6) suggested a functional role of GSLs in...
cell growth control. GM3 and other gangliosides were later shown to affect growth factor receptor function through inhibition of tyrosine kinases associated with the receptors (Refs. 35 and 36; for review, see Ref. 37). Recently GM3 complexed with tetraspanin CD9 was found to inhibit integrin-dependent cell motility (25, 28). These studies suggest that gangliosides interact with these key membrane components and thereby provide a basis for cell growth control.

A number of studies during the past decade indicate that specific microdomains at the cell surface play essential roles in signal transduction (38, 39). Some microdomains share the functions of adhesion receptors (e.g. integrin with tetraspanin) (25, 28), growth factor receptors (40, 41), and caveolae involved in endo- or exocytosis (38). Microdomains involved in cellular interaction/adhesion through GSLs (42) or O-linked or N-linked glycoproteins (43) coupled with signal transduction were termed "glycosynapses" (44) in analogy to "immunological synapse" (45). GEM in WI38 and VA13, in the present study, control cellular interaction and signaling and are therefore a type of glycosynapse (44). They are distinct from "caveolae" or "lipid raft," which are not involved in cellular interaction/adhesion and whose signaling function depends highly on cholesterol and other lipids (38, 46, 47).

Our previous studies on the functional role of GEM in defining the phenotype of melanoma (17, 18) and colorectal cancer cells (25, 28) suggest that certain tumor cell phenotypes, such as aberrant cell adhesion/motility and signal transduction, result from compositional and functional changes of GEM. However, properties of these tumor cell lines studied were not compared with those of progenitor cells since non-transformed progenitor cells of melanoma or colorectal cancer were not available. In the present study, functional organization of GEM components from human embryonal lung fibroblast WI38 and its SV40 transformant VA13 were compared. Our views on control of cell growth by specific components in GEM of WI38 versus VA13 cells are described below.

Properties of WI38 and VA13 GEM: Their Composition and Distinction from Caveolar Membrane or Lipid Raft—Contrasting composition of GEM from WI38 versus VA13 and resistance of components to Brj98, Triton X-100, β-CD, and saponin, as shown in Figs. 2 and 3, indicate the following. (i) Some components in WI38 GEM were less resistant than those in VA13; e.g. FGFR in VA13 was resistant to both Brj98 and Triton X-100, whereas FGFR in WI38 was solubilized in Triton X-100. (ii) All components in VA13 were resistant to β-CD, whereas FGFR, cSrc, Csk, and CD9 in WI38 were slightly or significantly reduced by β-CD. (iii) Caveolin, GM3, and CD81 in both WI38 and VA13 were completely resistant to β-CD. This property was distinct from that of caveolar membrane in which caveolin is highly susceptible to β-CD (38). Microdomain composed of an integrin-tetraspanin complex was solubilized in 1% Triton X-100 but resistant to 1% Brj98 and to pretreatment with β-CD (48). Our previous studies indicate that integrin-tetraspanin-ganglioside complex is resistant to 0.25 or 0.5% Triton X-100 (49) and to 1% Brj98 (25, 27, 28). This complex was also resistant to 10–15 mM β-CD.2 It is possible that the stability of microdomain containing a high level of tetraspanin

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2 Y. Kawakami and S. Hakomori, unpublished data.
is less dependent on cholesterol or other lipids and is not disrupted by β-CD. GM3 and other gangliosides are highly enriched in GEM regardless of type of detergent applied (15, 17) and are separable from caveolae (18).

**GM3-dependent Control of WI38 Cell Growth—**WI38 cell growth control was studied using three criteria: (i) GM3-dependent control as probed by P4 treatment, (ii) FGFR-induced MAPK activation and its promotion by GM3 depletion, and (iii) correlation of criteria i and ii with actively growing sparse culture versus growth-restricted confluent culture. Results of these studies clearly indicate that GM3 inhibits FGFR-induced MAPK activation and that GM3 depletion by P4 enhances MAPK activation. The degree of GM3-dependent inhibition of MAPK activation was higher in confluent than in sparse culture (Fig. 5A, W, row 6, S versus C). Expression of inactive cSrc, characterized by Tyr-527, was also higher in confluent than in sparse culture (Fig. 5A, row 4, S versus C). It is clear from these findings that interaction of components in microdomain is different between actively growing versus confluent culture of WI38, in particular GM3 interaction with and inhibition of FGFR, which is probably promoted by tetraspanin CD9 or CD81 (see Fig. 10).

Enhanced expression of inactive cSrc or Src family kinase, characteristic of Tyr-527 in confluent culture, is closely associated with the presence of Csk, the physiological inhibitor of the Src family kinases. Csk is characterized by a C-terminal kinase domain that has high homology with that of cSrc and Src family kinases but lacks Tyr-416. The inhibitory effect of Csk is due to its capability to phosphorylate C-terminal Tyr-527 of human cSrc, Tyr-507 of Lyn, or Tyr-505 of p56lck (50–52), which inhibits conformational change leading to cSrc activation with Tyr-416 phosphate (53). The presence of Csk in WI38 GEM, particularly in confluent culture, enhances expression of inactive cSrc and thereby decreases the level of activated cSrc. It is noteworthy that this process is also inhibited by GM3. FGFR activation in GEM can be relayed through the well-known SH2/SH3/Sos/Raf/MAPK pathway (54). A proposed scheme for the growth control process in WI38 GEM is illustrated in Fig. 10A.

**GEM Components in VA13 and Loss of Cell Growth Control—**Transformed VA13 in contrast to WI38 cells display constitutive activation of cSrc and MAPK regardless of FGFR stimulation, GM3 depletion, or cell population density. The strong cSrc activation is ascribable to (i) a high level of cSrc in GEM, (ii) the absence of inhibitory Csk, and (iii) a very high level of FGFR, compared with WI38, that promotes cSrc activation since FGFR interacts directly with cSrc as indicated by co-IP data. The strong MAPK activation in VA13 may be caused by (i) high levels of both cSrc and FGFR compared with WI38, (ii) an unchanged GM3 level, which is insufficient to inhibit FGFR-induced signaling, or (iii) reduced GM3-FGFR interaction resulting from a much lower level of CD9/CD81. Interaction of GM3 with FGFR in GEM at the surface of two interfacing cells (trans interaction) may also be reduced in VA13 because the CD9/CD81 level is minimal and Csk is absent, signaling through FGFR to cSrc (1) and that through clustered GM3 to cSrc (2) is relayed to MAPK without interruption (3). The inhibitory effect of GM3 on FGFR is nearly absent since the CD9/CD81 level is low. Thus, FGFR-dependent activation of FGF through cSrc is relayed to MAPK (3), and both activated cSrc and MAPK are continuously present regardless of cell contact. trans interaction between FGFR and GM3, as indicated by dashed arrows, is weaker than in WI38. Levels of FGFR and cSrc in VA13 GEM are much higher than in WI38 as reflected by the size of the shapes and symbols.

**FIG. 10. Components and their possible role in GEM of WI38 versus VA13 cells.** Possible growth control mechanisms of WI38 versus VA13 cells are illustrated based on differences in components and signal transducer activity. A, in WI38 cells (i) FGFR causes FGFR activation and minimal signaling (2) through its association with FGFR and cSrc, and consequently minimal MAPK activation (5). This lack of response is due to the fact that the FGFR level in WI38 is low (compared with VA13), and GM3 inhibits FGFR activity (4). This inhibitory effect of GM3 may be enhanced by formation of a complex with CD9/CD81. (ii) GM3 clustering, or GM3 interaction with FGFR, may occur upon cell contact, which in turn activates cSrc (1, 2). However, activation of cSrc is minimal because Csk, the physiological cSrc inhibitor (3), is associated in the same microdomain. Which molecular interactions with GM3 upon cell contact (i.e. trans interaction between interfacing GEM) to induce contact inhibition is unknown. FGFR was shown to interact preferentially with GM3 based on direct binding of FGFR to GM3-coated beads and data from confocal microscopy. Such trans interaction is stronger in WI38 than in VA13. B, in VA13 cells, because the CD9/CD81 level is minimal and Csk is absent, signaling through trans interaction to cSrc (1) and that through clustered GM3 to cSrc (2) is relayed to MAPK without interruption (3). The inhibitory effect of GM3 on FGFR is nearly absent since the CD9/CD81 level is low. Thus, FGFR-dependent activation of FGF through cSrc is relayed to MAPK (3), and both activated cSrc and MAPK are continuously present regardless of cell contact. trans interaction between FGFR and GM3, as indicated by dashed arrows, is weaker than in WI38. Levels of FGFR and cSrc in VA13 GEM are much higher than in WI38 as reflected by the size of the shapes and symbols.

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