Effect of Ganglioside and Tetraspanins in Microdomains on Interaction of Integrins with Fibroblast Growth Factor Receptor*  

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The functional interaction ("cross-talk") of integrins with growth factor receptors has become increasingly clear as a basic mechanism in cell biology, defining cell growth, adhesion, and motility. However, no studies have addressed the microdomains in which such interaction takes place nor the effect of gangliosides and tetraspanins (TSPs) on such interaction. Growth of human embryonal WI38 fibroblasts is highly dependent on fibroblast growth factor (FGF) and its receptor (FGFR), stably associated with ganglioside GM3 and TSPs CD9 and CD81 in the ganglioside-enriched microdomain. Adhesion and motility of these cells are mediated by laminin-5 (LN5) and fibronectin (FN) through α3β1 and α5β1 integrin receptors, respectively. When WI38 cells or its transformant VA13 cells were adhered to LN5 or FN, α3β1 or α5β1 were stimulated, giving rise to signaling to activate FGFR through tyrosine phosphorylation and inducing cell proliferation under serum-free conditions without FGF addition. Types and intensity of signaling during the time course differed significantly depending on the type of integrin stimulated (α3β1 versus α5β1), and on cell type (WI38 versus VA13). Such effect of cross-talk between integrins and FGFR was influenced strongly by the change of GM3 and TSPs. (i) GM3 depletion by P4 caused enhanced tyrosine phosphorylation of FGFR and Akt followed by MAPK activation, without significant change of ceramide level. GM3 depletion also caused enhanced co-immunoprecipitation of FGFR with α3/α5β1 and of these integrins with CD9/CD81. (ii) LN5- or FN-dependent proliferation of both WI38 and VA13 was strongly enhanced by GM3 depletion and by CD9/CD81 knockdown by siRNA. Thus, integrin-FGFR cross-talk is strongly influenced by GM3 and/or TSPs within the ganglioside-enriched microdomain.

Cell growth and associated differentiation are controlled by growth factors, and their receptors that have tyrosine kinases at the cytoplasmic domain (1–3). Cell adhesion to extracellular matrix and motility are mediated by integrin receptors consisting of various combinations of α and β subunits specific for extracellular matrix components, laminin (LN),1 fibronectin (FN), and collagen (4, 5). In contrast, some integrins on hematopoietic cells mediate cell-to-cell interaction based on their binding capability to Ig-like receptors (ICAM-1, VCAM-1) (6).

Growth factor receptors (GFRs) and integrin receptors clearly differ in their domain structure, mechanism of generating signaling, and localization in the membrane microdomain. Epidermal GFR (EGFR) and platelet-derived GFR are claimed to be associated with caveolar membrane (7, 8), whereas integrin receptors are soluble in 1% Triton X-100 and are considered to be located outside of the caveolar membrane or "raft" (9). However, in recent studies, integrin receptors have often been found associated with tetraspanins (TSPs) (10, 11), and integrin-TSP complexes are insoluble in 0.5% Triton X-100 (12) or in 1% Brij 98 (10, 13–16). Gangliosides are capable of interacting with and modulating the function of various GFRs (17–19), integrins (20, 21), Src family kinases, small G-proteins, and various other signal transducers (for review see Refs. 22 and 23). Glycosphingolipids (GSLs) in general have been implicated as inhibiting signal transduction, because various signaling molecules (e.g. c-Src, phospholipase Cγ) are activated when GSLs are depleted (24, 25) by GlcCer synthase inhibitors (26) (see "Discussion").

Since the discovery of integrin-induced EGFR activation (27), “cross-talk” between integrins and GFRs has been a hot topic in various aspects of cell biology (28) (for review see Ref. 29). However, the possible effect on such cross-talk of gangliosides, TSPs, or other membrane components in microdomains has not yet been studied. We previously observed that the GEM fraction of WI38 cells prepared in 1% Brij 98 contains stably associated FGFR and high levels of TSPs CD9 and CD81, in addition to c-Src, Csk (C-terminal Src kinase), and Lyn (19). Similar GEM components were found in VA13, although their proportions differed greatly; i.e. the VA13 GEM fraction contained much higher (4–5-fold) levels of PGFR and c-Src, a much lower level of TSP (only 15–20%) than WI38 GEM, and an undetectable level of Csk.

In the present study we found integrins α3β1 and α5β1 in WI38 and VA13 GEM prepared in 1% Brij 98, in addition to the above components. We also found a striking functional interaction between integrins and FGFR and studied the effects of ganglioside GM3, TSPs CD9/CD81, and receptor N-glycosyla-
tion on such interaction. Applying P4 (to deplete GM3), RNA interference (to knock down CD9/CD81), and N-glycosylation processing inhibitor 1-deoxynamomijirimycin (DMJ), we found that GM3 and/or CD9/CD81 strongly affect the functional interaction between integrins and FGFR and, consequently, affect FGF-independent proliferation in both WI38 and VA13 cells. GM3, associated with CD9/CD81 or with FGFR, may inhibit integrin-induced signaling through Akt, c-Src, and MAPK to activate FGFR. The exact mechanism of this signaling pathway remains to be studied, but ceramide may not be involved, since GM3 depletion by P4 does not significantly change ceramide level.

MATERIALS AND METHODS

Cells, Antibodies, and Reagents

Cells—Human embryonal lung diploid fibroblast WI38 and its SV40-transformed cell line VA13 were from American Type Culture Collection (ATCC, Manassas, VA). WI38 passage numbers 18–26 were used for experiments. WI38 and VA13 were grown in minimum essential medium (MEM, In Vitro) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 1 mM sodium pyruvate, and penicillin/streptomycin at 37 °C, 5% CO2. LN5-producing HDEF cells, anti–(Tyr653–Tyr654) rabbit Ig, anti-P-Akt (Ser473) rabbit IgG, and anti-Akt rabbit IgG were from Santa Cruz Biotechnology, Santa Cruz, CA; anti-c-Src (SRC2) rabbit IgG, anti-caveolin-1 rabbit Ig, and anti-FGFR-3 rabbit IgG were from Santa Cruz Biotechnology.

Antibodies—Anti-P-MAPK (Thr202/Tyr204) rabbit IgG, anti-MAPK (p42/44) rabbit IgG, anti-P-Src (Tyr416) rabbit IgG; anti-P-FGFR (Tyr555–Tyr565) rabbit IgG, anti-P-Akt (Ser473) rabbit IgG, and anti-Akt rabbit IgG were from Cell Signaling Technologies, Beverly, MA; anti-c-Src (SRC2) rabbit IgG, anti-caveolin-1 rabbit Ig, and anti-FGFR-3 rabbit IgG were from Santa Cruz Biotechnology, Santa Cruz, CA; anti-FGFR (Ab-1) mouse IgG, was from Oncogene Science, Cambridge, MA; anti-α3 rabbit IgG, anti-α5 rabbit IgG, and anti-β1 rabbit IgG were from Chemicon, Temecula, CA, anti-Cd81 mouse IgG2a from Beckman Coulter, Brea, CA, and anti-Cd9 mouse IgG2a from BD Bioscences; anti-β-actin mouse IgG was from Sigma; goat anti-rabbit IgG-horseradish peroxidase was from Transduction Laboratories, Lexington, KY, and goat anti-mouse IgG-horseradish peroxidase from Santa Cruz Biotechnology.

Reagents—FN from human plasma, poly-l-lysine, DMJ, and swainsonine were from Sigma; siRNA-Cd9 and siRNA-Cd81 were from Ambion; TX; d-serine-1-phenyl-2-palmitylaminolysine-3-p-anilino-1-propanol (P4) was kindly donated by Dr. James A. Shayman, University of Michigan; and d-erythro-MAPP was from Matreya, Pleasant Gap, PA. Other reagents were from Sigma unless indicated otherwise.

Preparation of Postnuclear Fraction and GEM

WI38 or VA13 (at least 5 × 10^6) cells cultured as described above were used for preparation of postnuclear fractions in 1% Brj 98 followed by sucrose density gradient centrifugation to isolate 12 fractions, including GEM (fractions 4–6), as described previously (15, 19). GSKs, TSPs CD9/CD81, FGFR and its activated form P-FGFR, integrins α5, α5, and β1, and various signal transducers (c-Src, Csk, Akt, MAPK) and their activated forms were determined by Western blot analysis as described previously (15, 19).

Preparation of Plates Coated with Extracellular Matrix Proteins (LN5, FN) or Poly-L-lysine as Control

LN5—HDEF cells grown as described above were harvested with 0.05% trypsin, 0.53 mM EDTA. This step was repeated twice to ensure that all HDEF cells were detached. Trypsin activity in plates and in cell suspension was inhibited by adding soybean trypsin inhibitor. FN—5 μg/ml FN in PBS was coated in bacterial plates (Fisher) overnight at 4 °C. Plates were washed with PBS, blocked with MEM-BSA, stored at 4 °C, and used within 2 days.

Poly-L-lysine—0.1% poly-l-lysine in PBS was coated in bacterial plates (Fisher) for 5 min at room temperature. Plates were washed with PBS, blocked with MEM-BSA, stored at 4 °C, and used within 2 days.

Activation of FGFR and Other Signal Transducers (c-Src, Akt, MAPK) following Stimulation of Integrin Receptors

Cells were grown in culture medium to ~95% confluence, washed with and starved overnight in serum-free MEM, and harvested with trypsin/EDTA. Trypsin activity was inhibited with soybean trypsin inhibitor (100 μg/ml). Cells were centrifuged and suspended in serum-free MEM at 5 × 10^6 cells/ml. To decrease basal phosphorylation of signaling molecules, cells were rotated for 1 h at 37 °C. A portion of this cell suspension was used as control cells without adhesion. The rest of the cells were added (2 ml suspension; 4 × 10^6 cells/cm^2) on 6-cm plates precoated with LN3, FN, or polyl-lysine as described above and incubated for various durations at 37 °C, to study the changes induced in FGFR activation and other signal transduction.

Cells were harvested by rubber scraper in ice-cold PBS containing sodium vanadate, centrifuged, and lysed in radioimmunoprecipitation assay buffer (30 mM HEPES, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM NaVO3, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 75 units/ml aprotinin). Lysate was collected after trypsinization at 2000 × g for 15 min. After protein quantification, ~30 μg of protein was loaded per well, and the degree of phosphorylation of standard signaling molecules was analyzed by Western blot using anti-P-FGFR, anti-c-Src, anti-Akt, and anti-P-MAPK antibodies.

Effect of Integrin-mediated Adhesion on FGFR-induced Cell Proliferation

LN5-, FN-, and polyl-lysine-coated 96-well plates were prepared as described above. WI38 and VA13 cells and cells were treated with adhesion with 1000 nM P4 (19, 30), incubation with 7500 nM DMJ, or co-transfection with 20 nM CD9 and CD9 siRNA. Nontreated cells adhered to LN5 or FN were used as control. For negative control of CD9/Cd81 siRNA transfectant, Silencer™ Negative Control #1 siRNA (Ambion; proprietary sequence) was transfected. All cells were starved overnight in serum-free MEM.

Cells were harvested with trypsin/EDTA, and trypsin was neutralized by soybean trypsin inhibitor. Cells were centrifuged, suspended in MEM-BSA (2 × 10^5 cells/50 μl), rotated at 37 °C for 1 h, and transferred (50 μl suspension) to the coated plates. After 30 min, 1.5 μCi of [H]thymidine was added per well.

After a 3-h incubation at 37 °C, cells were washed five times with PBS, harvested with trypsin/EDTA, collected in Eppendorf tubes, centrifuged at 3000 rpm, and suspended in scintillation liquid. Incorporation of [H]thymidine was measured by scintillation counter. Four independent experiments were conducted.

Effect of GM3 Level and Receptor N-Glycosylation on Cross-talk between Integrin and FGFR

WI38 and VA13 (2 × 10^6 cells) were grown in culture medium in 150-mm plates. After 16 h at 37 °C, the medium was replaced by fresh complete medium with or without 1000 nM P4, 7500 nM DMJ, or 7500 nM swainsonine for 24 h. Cells were adhered to LN5 or FN were used as control. For negative control of CD9/Cd81 siRNA transfectant, Silencer™ Negative Control #1 siRNA (Ambion; proprietary sequence) was transfected. All cells were starved overnight in serum-free MEM.

Preparation of Plates Coated with Extracellular Matrix Proteins (LN5, FN) or Poly-L-lysine as Control

LN5—HDEF cells grown as described above were harvested with 0.05% trypsin, 0.53 mM EDTA. This step was repeated twice to ensure that all HDEF cells were detached. Trypsin activity in plates and in cell suspension was inhibited by adding soybean trypsin inhibitor (In Vitro) at 100 μg/ml in PBS. Plates were washed twice with PBS, blocked with serum-free MEM containing 1% heat-inactivated mouse IgG at 60 °C BSA (MEM-BSA), stored at 4 °C, and used within 2 days.

FN—5 μg/ml FN in PBS was coated in bacterial plates (Fisher) overnight at 4 °C. Plates were washed with PBS, blocked with MEM-BSA, stored at 4 °C, and used within 2 days.

Poly-L-lysine—0.1% poly-l-lysine in PBS was coated in bacterial plates (Fisher) for 5 min at room temperature. Plates were washed with PBS, blocked with MEM-BSA, stored at 4 °C, and used within 2 days.
original WI38 and VA13 and in those co-transfected with siRNA was determined by Western blot with 10 μg of protein. Silencer<sup>®</sup> Negative Control #1 siRNA (Ambion) was used as negative control, under the same conditions as for CD9 and CD81 siRNA.

**Effect of Treatment with Various Concentrations of P4 for Various Durations on Integrin-induced FGFR Tyrosine Phosphorylation**

Cells were grown as described under "Cells, Antibodies, and Reagents." Different groups were treated with P4 for 24, 48, or 72 h. For the 24-h treatment, subconfluent cells were cultured in serum-free MEM containing P4. For the 48-h treatment, cells were cultured in medium with serum and then in medium without serum, both containing P4 and both for 24 h. For the 72-h treatment, cells were cultured in medium with serum for 48 h and then without serum for 24 h, with both media containing P4. Within each group, subgroups were treated with 0, 100, or 1000 nm P4. All subgroups were subjected to a determination of ceramide quantity, as described in the following section. Subgroups from each treatment condition were subjected to determination of integrin-induced FGFR tyrosine phosphorylation by Western blot analysis with antibodies to tyrosine-phosphorylated (Tyr<sup>553</sup>-Tyr<sup>654</sup>) FGFR as described above. The protein content applied in each Western blot was ~10 μg, rather than 30 μg. This condition was required to distinguish the occurrence of FGFR activation under different P4 treatments.

**Determination of Relative Ceramide Quantity**

Cultured cells treated with P4 at various concentrations and durations (see above) were subjected to determination of relative ceramide quantity compared with nontreated cells. Cells (3 × 10<sup>6</sup>) were extracted in chloroform/methanol/water (2:1:0.8, v/v/v) by vigorous agitation. Chloroform and water (1 ml each) were added, mixed, and centrifuged (3000 rpm, 5 min) to separate the upper from the lower phase (32). Total phospholipids in the lower phase were determined by Scion densitometric analysis of blot from Dittmer-Lester reagent (33) (Sigma) as described previously (34). Quantity of ceramide in the lower phase was determined as ceramide-1-[<sup>32</sup>P]phosphate following <sup>32</sup>P-phosphorylation by diacylglycerol kinase (Calbiochem), precisely following the method of Bektas et al. (35). C<sub>16</sub>-ceramide (Matreya) was used as control. Ceramide-1-[<sup>32</sup>P]phosphate was separated on high performance TLC developed in chloroform/acetone/methanol/acetic acid/water (10:4:3:2:1,v/v/v/v) (35). The relative amount of ceramide-1-[<sup>32</sup>P]phosphate from P4-treated versus nontreated cells, using aliquots with the same quantity (30 nmol) of total phospholipid, was quantified as digital light units (DLU) using a phosphorimaging device (Cyclone Storage Phosphor Screen, PerkinElmer Life Sciences), and expressed as the ratio of DLU to 30 μg. This condition was required to distinguish the occurrence of FGFR activation under different P4 treatments.

**Distribution Pattern of P4 in Cells Incubated with P4**

WI38 or VA13 cells treated with 100 or 1000 nm P4 for 48 or 72 h were harvested. The postnuclear fraction was prepared and separated into 12 fractions by sucrose density gradient centrifugation as described above. Lipids present in each fraction were adsorbed on C18 silica gel columns (Varian), separated from sucrose and other water-soluble components, eluted in chloroform/methanol 2:1, separated on TLC under conditions similar to those for ceramide, and detected by primulin spray. Fluorescence intensity corresponding to standard P4 was estimated as pmol/nmol phospholipid.

**RESULTS**

**WI38 Cell Adhesion to LN5- or FN-coated Plates Induces Activation of FGFR and Other Signal Transducers—**When WI38 cells were adhered to LN5- or FN-coated plates, FGFR was activated, as indicated by its tyrosine phosphorylation (at Tyr<sup>553</sup>-Tyr<sup>654</sup>) (Fig. 1A, columns a and b, row 1). FGFR activation occurred within 10 min to 1 h. FGFR activation was not observed in cell suspension without cell adhesion (Fig. 1A, column S, row 1) or in cells adhered on poly-l-lysine-coated plates (data not shown).

In close association with FGFR activation, strong c-Src activation also occurred during the early stage (10 min to 1 h) of cell adhesion to LN5 or FN (Fig. 1A, columns a and b, row 3); Akt activation after adhesion to FN also occurred at the early stage (10 min to 1 h), whereas Akt activation after adhesion to LN5 was observed in the later stage (10 min to 1 h).

**Interaction of Integrins with FGFR**

**FIG. 1.** FGFR activation and associated changes of c-Src, Akt, and MAPK in human embryonic fibroblast WI38 and its SV40 transformant, VA13, induced by adhesion of these cells to LN5- or FN-coated plates. A, WI38 cell suspensions (~1 × 10<sup>6</sup> cells/2 ml of MEM-BSA) were prepared and placed on LN5-coated (a) or FN-coated (b) plates (6-cm diameter) or as cell suspensions without adhesion (S), as described under "Materials and Methods." Cells that adhered for 10 min, 30 min, 1 h, 4 h, or 24 h were analyzed for activated (tyrosine-phosphorylated) FGFR (P-FGFR) (row 1), FGFR quantity (row 2), activated Src kinase (P-Src) (row 3), c-Src quantity (row 4), activated (tyrosine-phosphorylated) Akt (P-Akt) (row 5), Akt quantity (row 6), activated MAPK (P-MAPK) (row 7), and MAPK quantity (row 8). β-Actin (row 9) was used as the loading control. Each component and its activated form as listed above were determined by a specific antibody as described under "Materials and Methods." B, VA13 cell suspensions were processed and incubated as described for A. Rows 1–9 are defined as in A, with determination by the same antibodies. In A and B, an equal protein quantity (~30 μg) of each fraction was analyzed. Similar results were obtained in five separate experiments. C, quantitative densitometry ratio of immunoblotted P-FGFR/FGFR from WI38 cells, shown in A. Intensity of band ratio was determined by Scion Image at different times of adhesion (10 min, 30 min, 1–24 h), dividing row 1 by row 2 in columns a and b of A. S represents cells in suspension without adhesion as described under "Materials and Methods." D, quantitative densitometry ratio of immunoblotted P-FGFR/FGFR from VA13 cells as shown in B. Intensity of band ratio was determined by Scion Image at different times of adhesion (10 min, 30 min, 1–24 h), dividing row 1 by row 2 in columns a and b of B. S represents cells in suspension without adhesion as described under "Materials and Methods."
LN5 occurred at a later stage (1–24 h) (Fig. 1A, row 5). In contrast, MAPK activation (P-MAPK) occurred at the later stage (1–24 h) regardless of adhesion to FN or LN5 (Fig. 1A, columns a and b, row 7). Only minimal activation was observed upon adhesion to poly-L-lysine (data not shown).

VA13 Cell Adhesion to LN5-or FN-coated Plates Induces Activation of FGFR and Other Signal Transducers—Adhesion of VA13 cells to LN5- or FN-coated plates induced activation of FGFR by tyrosine phosphorylation (Fig. 1B, columns a and b, row 1) similar to that in WI38 cells. However, the pattern of associated signal transducer changes differed from that in WI38. c-Src activation occurred strongly at a later stage, i.e. 24 h after adhesion to both LN5 and FN (Fig. 1B, columns a and b, row 3). Akt activation was stronger when cells were adhered to FN than to LN5 and was higher at the early stage (10 min to 1 h) (Fig. 1B, column b, row 5). In contrast, MAPK activation was stronger when cells were adhered to LN5 than to FN and in both cases was higher at the later stage (4–24 h) (Fig. 1B, columns a and b, row 7).

Quantitative Time Course Change of FGFR Phosphorylation Induced by Integrin-dependent Adhesion by WI38 and VA13 Cells on LN5 of FN—Because FGFR activation in terms of Tyr653/Tyr654 phosphorylation induced by LN5- or FN-dependent adhesion is of primary importance, the time course process was determined quantitatively as described in the legend for Fig. 1, C and D. The results indicate that FGFR activation occurred consistently at the early stage (10 min to 1 h) after cell adhesion to LN5 or FN in both WI38 and VA13 cells.

Change of GM3 Level Alters Functional Interaction of Integrin with FGFR in WI38 and VA13 Cells—Ganglioside GM3 is the sole GSL present in low-density microdomain fraction of WI38 cells. ~95% of total GM3 was depleted upon preincubation of cells with P4 (1000 nm, 72 h) (19). Depletion of gangliosides by P4, at similar concentration and duration, had been observed previously in other types of cells (30). A lower concentration of P4 or a shorter incubation time resulted in a lesser depletion of GM3 (see below). GM3 suppressed the process of LN5-induced FGFR activation, because depletion of GM3 by P4 significantly enhanced FGFR tyrosine phosphorylation. Such differential effect on FGFR phosphorylation in the presence versus absence of GM3 was more clear in LN5-induced than in FN-induced adhesion of WI38 cells (Fig. 2A, row 1 versus 3). A similar differential effect in the presence versus absence of GM3 was observed for Akt phosphorylation (Fig. 2A, row 5 versus 7).

In contrast, in transformed VA13 cells, the presence of GM3 versus its depletion by P4 made no clear difference in the effect of integrin-induced activation on FGFR tyrosine phosphorylation (Fig. 2B, row 1 versus 3) or on Akt phosphorylation (row 5 versus 7). However, VA13 showed much higher MAPK phosphorylation upon adhesion to LN5 or FN when GM3 was depleted by P4, versus in the presence of GM3 (Fig. 2B, row 9 versus 11). This high level of activated MAPK (P-MAPK), even in the absence of P4 treatment, is presumably because the FGFR level is much higher in VA13 than in WI38 cells and the quantity of GM3 in VA13 is not sufficient to inhibit FGFR-induced signaling leading to MAPK activation.

GM3 Depletion Causes Change of Co-immunoprecipitation Pattern between FGFR and Integrins and among Integrin Subunits, Indicating That GM3 Modulates Interaction of These Components in GEM—The effect of GM3 on the interaction between FGFR and integrin and on the interaction among integrin subunits in WI38 cells was determined by the co-immunoprecipitation pattern as described under “Materials and Methods.” The degree of co-immunoprecipitation between FGFR and α3/α5 was significantly higher in P4-treated WI38 cells in which GM3 was depleted (Fig. 3, column b, rows 3 versus 4 and 5 versus 6). FGFR co-immunoprecipitated with c-Src was significantly higher in P4-treated cells (Fig. 3, column b, row 10 versus 9). α3 or α5 was co-immunoprecipitated strongly with β1 only in P4-treated cells (Fig. 3, column c, row 8 versus 7). The degree of co-immunoprecipitation of α3 with CD9 or with CD81 was slightly higher in P4-treated cells (Fig. 3, column c, rows 11 versus 12 and 13 versus 14).

These results suggest that GM3 may inhibit the interaction of FGFR with α3 and α5, as well as with c-Src, since GM3 depletion enhances such interactions, even though GM3 by itself interacts specifically with FGFR as described previously (19) (see “Discussion”).

Effect of P4 Concentration and Treatment Duration on Ceramide Level and on Integrin-induced FGFR Tyrosine Phosphorylation—The relative ceramide level in cells treated with 100 or 1000 nm P4 for 24, 48, or 72 h, as compared with the level in control cells (treated with 0 nm P4 for 24, 48, or 72 h) (defined as 1.0), was always within the 0.8 to 1.2 range (Table I, under column heading “Relative ceramide level”). This relative level was determined as ceramide-1-[^32]P phosphate and expressed as the ratio of DLU in P4-treated versus nontreated cells based on the same phospholipid quantity (30 nmol) (see “Materials and Methods”). However, the relative ceramide level increased significantly (i.e. the DLU ratio became 1.7–1.8) when these cells were treated with the ceramidase inhibitor d-erythro-MAPP for 24 h (Table 1).

Enhanced tyrosine phosphorylation of FGFR induced by LN5- or FN-dependent adhesion was observed only when WI38 or VA13 cells were treated with 1000 nm P4 for 72 h, using ~10 μg of protein quantity/ lane for Western blot. Enhanced FGFR
tyrosine phosphorylation was not observed for cells treated with 0 or 100 nM P4 (Fig. 4, A and B).

GM3 was >95% depleted (i.e., 2-5% of original amount remained) only when cells were treated with 1000 nM P4 for 72 h. Other conditions (100 or 1000 nM P4 for 48 h, or 100 nM P4 for 72 h) resulted in a lesser depletion of GM3 (i.e. 20-70% was lost) (Table I, column “GM3 level”).

Association of P4 with Membrane in P4-treated Cells—WI38 or VA13 cells treated with P4 were separated into 12 fractions, and P4 in each fraction was determined by TLC as described under “Materials and Methods.” The majority of P4 adsorbed to the cells (i.e. 10-20% of total P4 added during cell culture) was found in fractions 1–3, the buoyant, lipid-rich fractions present above the GEM fractions. Only a very small quantity (<1% of total) of P4 added was found in fractions 4–6 (i.e. GEM). No P4 was detectable in the high density, protein-rich fraction (fractions 7–9 and 10–12), and 14 are from GM3-depleted (P4-treated) cells. An equal protein quantity (~30 µg) of each fraction was analyzed. Similar results were obtained in three separate experiments.

DISCUSSION

Cell growth is regulated by growth factors and their receptors with associated tyrosine kinases (e.g. see Refs. 1–3), whereas cell adhesion and motility are controlled primarily by integrin receptors consisting of various combinations of α and β subunits (4, 5). These two types of receptors clearly differ in their structure, mechanism for generating signaling upon binding to ligand, and localization in the membrane microdomain. EGFR and platelet-derived GFR are claimed to be transiently associated with the caveolar membrane, or raft, upon activation (7, 8). In contrast, integrin receptors are found outside of the caveolar membrane or raft and are soluble in 1% Triton X-100 (9).

The ganglioside-enriched microdomain in some types of cells, separated in 1% Brij 98, contains a high level of TSP, integrin, and/or GFR. GSLs, particularly gangliosides, may mediate formation of TSP complex with integrin, or with GFR. Gangliosides in such complex control TSP/integrin-dependent cell motility (15) or TSP/GFR-dependent cell growth (19). A striking feature of such GEM is its resistance to cholesterol-binding reagents such as β-cyclodextrin (19), indicating that assembly of GFR or integrin in GEM does not require a high level of cholesterol but may require a high level of TSP. This cast doubt on the concept of lipid rafts, which claims that all detergent-resistant microdomains are cholesterol-dependent (37–39). Such GEM, enriched in glycoconjugates, is often involved in carbohydrate-dependent or -modulated cell adhesion and signaling and has been termed “glycosynapse” (40–42), in analogy to “immunological synapse” (43, 44).

In 1998, a close reciprocal interaction between integrin β1 and EGFR was reported in breast cancer cells under three-dimensional culture (27). Since then, there have been many studies on the functional interaction (cross-talk) of integrins with GFRs (e.g. in Ref. 28; for review see Ref. 29). However, two major points regarding the mechanism of the integrin-to-GFR interaction have not been addressed: (i) characterization of the microdomain in which integrin interacts with GFR and types of signaling molecules involved; (ii) effects of gangliosides, TSPs, and N-glycosylation status of the receptors involved on cross-talk between integrin and GFR.

The present study, which focused on these two questions, had the following findings. (i) Integrins α3/α5/β1 are present in WI38 and VA13 GEM prepared in 1% Brij 98, although the microdomain associated with integrin and that associated with FGFR are not easily separated, nor have they been differentially identified thus far. (ii) c-Src, Akt, and MAPK in the microdomain are activated following integrin stimulation that leads to FGFR activation. (iii) Stimulation of α3β1, upon adhesion of cells to LN5, and that of α5β1, upon adhesion of cells to FN, both induce FGFR activation in the absence of GFG or under serum-free conditions. (iii) The activation status of signal transducers following integrin stimulation differs depending on duration and on type of integrin involved. (iv) Depletion of
FGFR band. No band was seen for cells not treated with P4.

FN-dependent cell adhesion. of WI38 or VA13 cells on FGFR activation induced by LN5- or 16232 anti-GM3 monoclonal antibody DH2.

FGFR with enhances interaction, in terms of co-immunoprecipitation, of or FN-dependent cell adhesion. (v) GM3 depletion by P4 also GM3 by P4 treatment enhances activation of FGFR upon LN5-

et al.). Significant proliferation enhancement was observed in both WI38 and VA13 cells adhered to LN5 or FN, particularly when cells were pretreated with P4 or siRNA for CD9/CD81.

GM3 by P4 treatment enhances activation of FGFR upon LN5- or FN-dependent cell adhesion. (v) GM3 depletion by P4 also enhances interaction, in terms of co-immunoprecipitation, of FGFR with o, FGFR with a, FGFR with b, a with b1, and a with TSP CD9/CD81.

Based on findings i–v above, we studied the degree of cell proliferation induced by LN5- or FN-dependent adhesion in cells treated with various methods (P4 for GM3 depletion, DMJ for N-glycosylation modification, siRNA for knockdown of CD9/CD81, etc.). Significant proliferation enhancement was observed in both WI38 and VA13 cells adhered to LN5 or FN, particularly when cells were pretreated with P4 or siRNA for CD9/CD81.

Levels of ceramide and GM3 in WI38 and VA13 cells treated with various concentrations of P4 for various durations

| Duration (h) | P4 concentration (nM) | Relative ceramide level | GM3 level |
|-------------|-----------------------|-------------------------|-----------|
|             |                       | WI38       | VA13      | WI38       | VA13      |
| 24          | 0                     | 1.0        | 1.0       | 100        | 100       |
| 24          | 100                   | 1.2        | 1.0       | 78–81      | 75–80     |
| 24          | 1000                  | 0.8        | 1.0       | 30–40      | 35–40     |
| 48          | 0                     | 1.0        | 1.0       | 100        | 100       |
| 48          | 100                   | 0.9        | 1.2       | 25–30      | 30–38     |
| 48          | 1000                  | 1.0        | 1.1       | 2           | 5         |
| 72          | 0                     | 1.2        | 0.8       | 2           | 2         |
| 72          | 100                   | 0.8        | 0.9       | 2           | 2         |
| 72          | 1000                  | 1.0        | 1.0       | 2           | 2         |
| Control (non-treated) |                   | 1.0        | 1.0       | 1.0        | 1.0       |
| d-erythro-MAPP (100 μM) |                   | 1.8        | 1.7       | 1.0        | 1.0       |

* Relative ceramide level was based on amount of ceramide-1-[^32]P phosphate from P4-treated versus nontreated cells using aliquots with equal 30 nmol of total phospholipid, quantified as absolute DLU using a phosphorimaging device, and expressed as DLU ratio (value in nontreated cells defined as 1.0) (see “Materials and Methods”).

* Expressed as percent relative to level in P4-nontreated cells (defined as 100%). GM3 level was determined by TLC immunostaining with anti-GM3 monoclonal antibody DH2.

The effects of GSLs on cellular function (e.g. cell growth, cell cycle) and associated signal transduction have been studied using GlcCer synthase inhibitor (initially PDMP, more recently P4). PDMP causes cell cycle arrest (45) and phospholipase C activation with inositol triphosphate release following bradykinin stimulation (46). These cellular responses may be ascribable in part to increased ceramide, because PDMP enhances the ceramide level through inhibition of GlcCer synthase (45) and ceramide is claimed to initiate multiple signaling pathways (47). Therefore, improved GlcCer synthase inhibitors P4 and its derivatives were subsequently developed, showing much less ceramide accumulation and greater GlcCer synthase inhibitory effect (26).

GSL depletion by d-ETDO-P4 (maximal effect at 100 nM) in human ECV304 cells (endothelial origin) activated phospholipase Cγ through its tyrosine phosphorylation and enhanced the release of inositol trisphosphate, which is associated with a high response to bradykinin (24). GSL depletion by d-ETDO-P4 in these cells also induced initial activation of c-Src, with enhanced trans-phosphorylation at Tyr418 coupled with dephosphorylation at Tyr249. This process was counteracted by the c-Src inhibitor 4-aminophenylketone.7-t-butylpyrazolo[3,4-d]pyrimidine (PP2). The process of GSL depletion and

![Table I: Levels of ceramide and GM3 in WI38 and VA13 cells treated with various concentrations of P4 for various durations](image_url)

![Fig. 4: Effect of P4 concentration and duration of incubation of WI38 or VA13 cells on FGFR activation induced by LN5- or FN-dependent cell adhesion.](image_url)

WI38 (A) or VA13 cells (B) were treated with 0, 100, or 1000 nM P4 for 48 or 72 h and then adhered for 30 min to plates coated with LN5, FN, or polylysine (P-Lys) as described under “Materials and Methods.” The levels of tyrosine-phosphorylated FGFR and of nonphosphorylated FGFR were determined by Western blot analysis, with anti-FGFR (5, 6, 7, 8) as positive control. The intensity of the bands was determined with "Materials and Methods). The intensity of the bands was determined by Western blot analysis, with ~10 μg of protein quantity/lane. The degree of expression, indicated below each band, is the ratio compared with β-actin (loading control). CD9 or CD81 expression level in VA13 was much lower (15–20%) than that in WI38 and was undetectable under this condition (but was detectable if Western blot was performed with a higher quantity of protein (19)).

![Fig. 5: Levels of CD9 and CD81 in WI38 cells before and after knockdown by siRNA approach.](image_url)

Total, expression of CD9 and CD81 in total WI38 cell extract as positive control. CD9/81 siRNA, expression of CD9 and CD81 (these TSP bands are inseparable under this condition) after co-transfection of siRNAs with specific sequences for CD9 and CD81 (see “Materials and Methods”). Neg C, negative control by transfection of Silencer™ Negative Control #1 siRNA (Ambion) (see “Materials and Methods”). The intensity of the bands was determined by Western blot analysis, with ~10 μg of protein quantity/lane. The degree of expression, indicated below each band, is the ratio compared with β-actin (loading control). CD9 or CD81 expression level in VA13 was much lower (15–20%) than that in WI38 and was undetectable under this condition (but was detectable if Western blot was performed with a higher quantity of protein (19)).
P4 added is found in GEM (fractions 4–6). It is difficult to correlate this distribution pattern with the functional significance of P4 in these fractions, since the same distribution pattern was found in cells treated with lower P4 concentration or shorter duration, whereby adhesion to LN5 or FN did not cause FGFR activation. The physiological significance of P4 in buoyant fractions versus GEM fractions is unclear, and clarification of this issue is beyond the scope of this study.

The possibility that heparan sulfate proteoglycan may mediate integrin cross-talk with FGFR was ruled out, because proteoglycan was undetectable in the GEM of these cells and desulfation in the presence of sodium chlorate (NaClO3) had no effect on FGFR-dependent MAPK activation in our previous studies (19).

GM3 is known to inhibit tyrosine kinases associated with GFRs (50, 51), including insulin-R (52–54) and FGFR (19). TSPs, particularly CD9, CD81, and CD9/CD82, may form complexes with GM3 that inhibit integrin function (14, 15). CD82 was recently shown to be associated with EGF-R in the microdomain and to inhibit EGF-dependent dimerization of EGF-R, thereby reducing EGF-induced signaling (55). It is therefore reasonable to assume that the following: (i) FGFR and/or integrins α5/β1 are stably associated with clusters of GM3 and CD9/CD82 in WI38 cell GEM; GM3 may mediate the interaction of TSP with integrin or with GFR in GEM. (ii) FGF-induced signaling through FGFR activation and cell adhesion-induced signaling through LN5/α3 or FN/α5 are modulated by GM3, or by GM3 complexed with CD9 or CD81. (iii) The functional interaction between α3/α5/β1 and FGFR is also modulated by GM3 alone or by GM3 complexed with appropriate TSP. Further studies based on these possibilities are in progress.

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