Enhancement of Epidermal Growth Factor Signaling and Activation of Src Kinase by Gangliosides*

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In a recent study, inhibition of cellular ganglioside synthesis blocked growth factor-induced fibroblast proliferation. Conversely, enrichment of cell membrane gangliosides by ganglioside preincubation enhanced growth factor-elicited cell proliferation. In the absence of serum and growth factors, NeuNacα2-3Galβ1-3GalNAcβ1-4(Galβ1-4Glcβ1-1Cer) (GD1a) acted like a growth factor when cells were pretreated with the ganglioside, stimulating proliferation of normal human dermal fibroblasts and Swiss 3T3 fibroblasts. In contrast, growth inhibition was observed when high concentrations of gangliosides were continuously present in the culture medium during incubation of fibroblasts with growth factors (Li, R., Manela, J., Kong, Y., and Ladisch, S. (2000) J. Biol. Chem. 275, 34213–34223). Here, we investigated the mechanisms whereby gangliosides elicit proliferation-coupled signaling in normal human dermal fibroblasts. Incubation of the fibroblasts with GD1a enhanced epidermal growth factor (EGF) receptor autophosphorylation and Ras and MAPK activation in a dose-dependent manner. Exposure of the cells to GD1a also enhanced the phosphorylation of Elk-1 by the activated MAPK. Brief pretreatment of the cells with PD98059 blocked the enhancing effect of gangliosides on EGF-induced MAPK activation. In the absence of serum and growth factors, GD1a incubation induced phosphorylation of Src kinase, Ras activation, and phosphorylation of MAPK and Elk-1 in a dose-dependent manner. The activation of Src kinase was confirmed by enhanced Src kinase activity. Brief treatment of the cells with PPI blocked the activation of Src kinase and MAPK. Again, PD98059 treatment inhibited ganglioside-elicited MAPK phosphorylation. Among the gangliosides tested, GD1α was the most active molecule, whereas lactosylceramide was the least active one, indicating relative structural specificity of the ganglioside action. In conclusion, gangliosides promote fibroblast proliferation through enhancement of growth factor signaling and activation of Src kinase.

Protein-tyrosine kinases are critical components of signaling pathways that control cell proliferation and differentiation. The receptors for most growth factors such as epidermal growth factor (EGF) are transmembrane tyrosine-specific protein kinases. They all share a crucial signaling pathway that runs from the cell surface to the nucleus. The binding of a growth factor to its tyrosine kinase receptor on the cell surface results in receptor trans autophosphorylation and activation of the Ras/Raf/MAPK cascade (1). This cascade passes the signal to the nucleus through phosphorylation of transcription factors that regulate gene expression, eventually leading to DNA synthesis and cell division (2, 3). In addition, the activated receptor tyrosine kinase may also activate other intracellular signaling proteins, including phospholipase Cγ, phosphatidylinositol 3-kinase, and Src-like non-receptor tyrosine kinase (1). Non-receptor tyrosine kinases, e.g. Src, have no extracellular or transmembrane domains but possess modular domains that are responsible for subcellular targeting and regulation of catalytic activity (4, 5). Both receptor tyrosine kinases and non-receptor Src family tyrosine kinases have been reported to be located in lipid rafts (5–7) or caveolae membrane domains (8, 9). Thus, an important issue is to investigate how membrane lipids interact with receptor molecules at the cell surface and initiate signaling for cells to proliferate.

Cell surface gangliosides exist in glycosphingolipid-enriched domains and modulate transmembrane signaling (10–12). Our recent study provides a new perspective on the biological effect of gangliosides on cell proliferation (13). Specific inhibition of cellular ganglioside synthesis that conceivably abolishes ganglioside domain formation blocks growth factor-mediated cell proliferation. Conversely, enrichment of cell membrane gangliosides by ganglioside incubation that conceivably promotes ganglioside domain formation enhances growth factor-elicited cell proliferation. Strikingly, gangliosides themselves exert a growth factor-like effect in the absence of serum and growth factors, enhancing fibroblast proliferation. However, the mechanisms remain to be investigated.

The purpose of this study is to determine how gangliosides stimulate fibroblast proliferation. We tested two specific hypotheses: (i) Interaction of gangliosides with EGF receptors in plasma membrane enhances EGF-receptor phosphorylation and MAPK activation. (ii) Gangliosides stimulate non-receptor Src kinase and initiate proliferation signaling. The present study demonstrates that gangliosides enhance epidermal growth factor signaling and activate Src kinase.

EXPERIMENTAL PROCEDURES

Materials—EGF (6 kDa, mouse submaxillary glands, receptor grade) was purchased from Sigma Chemical Co. (St. Louis, MO) and serum; NHDF, normal human dermal fibroblasts; MAPK, mitogen-activated protein kinase; Erk2, extracellular signal-regulated kinase 2; MEK, MAPK/ERK kinase; FGM, fibroblast growth medium; FBM, fibroblast basal medium; PAGE, polyacrylamide gel electrophoresis; HPTLC, high performance thin-layer chromatography; Csk, carboxy-terminal Src kinase.

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‡ The abbreviations: EGF, epidermal growth factor; FBS, fetal bovine serum; NHDF, normal human dermal fibroblasts; MAPK, mitogen-activated protein kinase; Erk2, extracellular signal-regulated kinase 2; MEK, MAPK/ERK kinase; FGM, fibroblast growth medium; FBM, fibroblast basal medium; PAGE, polyacrylamide gel electrophoresis; HPTLC, high performance thin-layer chromatography; Csk, carboxy-terminal Src kinase.

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reconstituted with serum-free medium. The stock solution was stored at -70 °C. EGF was directly added to the culture medium by diluting the stock solutions before use. GmA3, Gb1b (bovine brain), Gm13, Gm16, and lactosylceramide (bovine buttermilk) were purchased from Matreya (Pleasant Gap, PA). Gangliosides and lactosylceramide were dissolved in dimethyl sulfoxide and stored at -20 °C. The dissolved medias used in the cultured for each assay. PD98059 (Sigma) and AG1478 and PPI (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) were dissolved in Me2SO and diluted directly to the culture medium in each assay (14, 15).

Phospho-p44/42 MAPK (Thr-202/Tyr-204) antibody (rabbit polyclonal IgG), p44/42 MAPK antibody (rabbit polyclonal IgG), p34-Ras (Ser-209) antibody (rabbit polyclonal IgG) were purchased from CLONTECH (San Diego, CA) and cultured in fibroblast growth medium (FGM) supplemented with 2% fetal bovine serum (FBS, HyClone, Logan, UT), 0.5 ml of insulin, 0.5 ml of human FGF, and 0.5 ml of GA1000 (Clonetics). For serum-free culture, fibroblast basal medium (FBM) was used. Cells from passages 3–10 were used for this study (13). The culture medium was changed every 3 days. Cell viability was assayed by trypan blue dye exclusion.

Preparation of Cell Lysate—NHDF were seeded at the density of 2 × 10^5 cells/dish (100 × 20 mm; area = 55 cm^2) or 1.5 × 10^5 cells/well in six-well plates (area = 9.4 cm^2) in FGM with 2% FBS. After incubation with ganglioside for 18 h, the cells were washed twice with serum-free medium and starved overnight (13, 16). The cells were then exposed to 2 ng/ml EGF in serum-free FBM for 5 min at 37 °C. In some experiments, cells were incubated with gangliosides either in FGM with 2% FBS or in serum-free FBM for 5 min, washed, and resuspended in serum-free FBM without starvation. The cells were immediately washed twice with ice-cold phosphate-buffered saline and lysed. The lysis buffer contained 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysate was transferred to microcentrifuge tubes, sonicated briefly on ice, and microcentrifuged for 10 min at 14,000 × g at 4 °C. The supernatant (the cell lysate) was adjusted to 1 μg/ml and used for kinase assays. Proteins were quantified by the Lowry method, using bovine albumin as a standard (17).

EGF Receptor Activation Assays—200 μl of cell lysate (~200 μg of total protein) was mixed with 100 μl of washed Protein G-Sepharose agarose bead slurry (50-μl packed beads) and stirred by a rotary shaker for 2 h at 4 °C to preclude nonspecific binding. After microcentrifugation at 14,000 × g for 5 s, the supernatant was transferred to a new microcentrifuge tube and mixed with 4 μg of anti-EGF receptor antibody (sheep polyclonal IgG). The mixture was incubated at 4°C overnight with stirring. The immune complexes were recovered by adding 100 μl of washed Protein G-Sepharose agarose bead slurry (50-μl packed beads) and gently rocking the mixture for 2 h at 4 °C. After microcentrifugation at 14,000 × g for 5 min and removal of the supernatant, the beads were washed three times with ice-cold lysis buffer, resuspended in 50 μl of 1× SDS sample buffer, and boiled for 5 min. After microcentrifugation, 20 μl of the supernatant of each sample (~40 μg) was loaded onto a 7.5% SDS-polyacrylamide gel. Phosphorylation of EGF receptor was detected by Western blot analysis using an anti-phosphotyrosine antibody p-Tyr (PY99). Alternatively, total cell lysate was used to determine the phosphorylation of EGF receptor, using either a phospho-EGF receptor antibody (Y1173, mouse monoclonal IgG) or an anti-phosphotyrosine antibody p-Tyr (PY99). Total EGF receptor was detected by an anti-ErbB1 antibody (Ab-2; Santa Cruz Biotechnology Inc.). Anti-phosphotyrosine antibody (PY99; mouse monoclonal IgG), anti-c-Src antibody (rabbit polyclonal IgG), and anti-Csk (C-20; rabbit polyclonal IgG) were obtained from Santa Cruz Biotechnology. Anti-phospho-Src-Ser-326 antibody (rabbit polyclonal IgG), purchased from BIOSOURCE International. ~P[32P]ATP (3000 Ci/mmol) was purchased from PerkinElmer Life Sciences.

Western Blotting—After transfer, the polyvinylidene fluoride membrane was incubated in 25 ml of blocking buffer for overnight at 4°C. The membrane was incubated with primary phospho-Erk1-1 (Ser-383) antibody (rabbit polyclonal IgG, 1:100 dilution) with gentle agitation overnight at 4°C. After being washed three times, the membrane was incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (1:2000) and proteins were detected using the LumiGLO chemiluminescence reagents. Proteins with different molecular weights were used as standards.

Western Blotting Assay—NHDF were seeded at the density of 2 × 10^5 cells/well (100 × 20 or 100 × 20 cm^2; area = 55 cm^2) or 1.5 × 10^5 cells/well in six-well plates (area = 9.4 cm^2) in FGM with 2% FBS and were cultured until their density reached ~70% confluence. The cells were then incubated with ganglioside in FGM with 2% FBS for 18 h. After removal of the culture medium by aspiration, the cells were washed twice with serum-free medium and starved overnight (13, 16). The cells were then exposed to 2 ng/ml EGF in serum-free FBM for 5 min at 37 °C. In some experiments, cells were incubated with gangliosides either in FGM with 2% FBS or in serum-free FBM for 5 min, washed, and resuspended in serum-free FBM without starvation. The cells were immediately washed twice with ice-cold phosphate-buffered saline and lysed. The lysis buffer contained 20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na2VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. The lysate was transferred to microcentrifuge tubes, sonicated briefly on ice, and microcentrifuged for 10 min at 4°C. The supernatant (the cell lysate) was adjusted to 1 μg/ml and used for kinase assays. Proteins were quantified by the Lowry method, using bovine albumin as a standard (17).

Ssrc Phosphorylation Assay—NHDF were seeded at the density of 2 × 10^5 cells/dish (100 × 20 cm^2; area = 55 cm^2) or 1.5 × 10^5 cells/well in six-well plates (area = 9.4 cm^2) in FGM with 2% FBS and were cultured until their density reached ~70% confluence. The cells were then incubated with ganglioside in FGM with 2% FBS for 18 h. After removal of the culture medium by aspiration, the cells were washed twice with serum-free medium and starved overnight (13), followed by the lysis of the cells as described above. In some experiments, cells were incubated with ganglioside either in FGM with 2% FBS for 6 or 18 h, washed, and lysed without serum starvation. The phosphorylation of Ssrc was detected by Western blot analysis, using phospho-Ssrc kinase antibody. Total Ssrc was measured by Western blot analysis using c-Src antibody.

Ssrc Kinase Activity Assay—Ssrc kinase activity was determined by measuring the phosphorylation of a Ssrc substrate peptide, using Ssrc kinase assay kit (Upstate Biotechnology Inc.). Briefly, Ssrc was immunoprecipitated from the cell lysate (~200 μg of protein). One-fifth of the immunoprecipitated protein (in 10 μl) was transferred to a microcentrifuge tube that contained 10 μl of Ssrc substrate peptide (final concentration, 120 μM) and 10 μl of Ssrc kinase reaction buffer (100 mM Tris-HCl, pH 7.2, 125 mM MgCl2, 25 mM MnCl2, 2 mM EGTA, 250 μM sodium orthovanadate, 2 mM dithiothreitol). After gentle mixing, 10 μl of ~P[32P]ATP (10 μCi) was added to the mixture. The mixture was incubated at 30 °C for 10 min with agitation, followed by addition of 20 μl of 40% trichloroacetic acid and incubation for 5 min at room temperature to precipitate the phosphorylated peptide. 25 μl of the mixture was then slowly spotted onto the center of a numbered P81 paper square. The paper squares were washed five times for 5 min with 0.5% phosphoric acid, dried once with air, and then dried for 3 min. Four paper squares were transferred to scintillation vials, and 5 ml of scintillation mixture was added for γ-counting. ~P incorporated into the substrate peptide was calculated by subtraction of the nonspecific binding of ~P[32P]ATP and binding of phosphorylated endogenous proteins in the sample extracts (B) from the total count (A). The picomoles of phosphate incorporated into substrate peptide per minute was calculated by the for-
mula, \((A - B) \times 2.4/\text{specific radioactivity} \times 10 \text{ min}\), where specific radioactivity = 5760 cpm/pmol of ATP.

Analysis of Csk—Csk in total cell lysate and plasma membrane was determined by Western blot analysis. Plasma membrane was isolated as previously described (19). NHDF at 90% confluence were washed twice with buffer consisting of 0.25 M sucrose/1 mM EDTA/20 mM Tricine, pH 7.8. The cells were scraped off the dishes in 3 ml of buffer. After being centrifuged at 1400 \(\times g\) for 5 min, cells from two culture dishes (100 \times 20 mm; area = 55 cm\(^2\)) were resuspended in 1.0 ml of buffer and homogenized in a 2-ml tissue grinder with 20 strokes. The suspension was then transferred to a 1.5 ml-centrifuge tube and centrifuged at 1000 \(\times g\) for 10 min. The postnuclear supernatant fraction was then used to detect EGF receptor phosphorylation (Fig. 1). GD1a preincubation clearly enhanced EGF receptor phosphorylation. Although exerting a strong enhancing effect on EGF receptor phosphorylation, ganglioside GD1a alone did not cause EGF receptor phosphorylation (Fig. 1A).

In the third approach, EGF receptors were first immunoprecipitated from the cell lysate with an anti-EGF receptor antibody. A specific anti-phosphotyrosine antibody, p-Tyr (PY99), was then used to detect EGF receptor phosphorylation (Fig. 1B). Again, ganglioside GD1a enhanced EGF receptor phosphorylation in the tested range of 10–50 \(\mu M\). The detection of EGF receptor phosphorylation using immunoprecipitated EGF receptors with a more sensitive approach than simply using the total cell lysate. For example, immunoprecipitation of NHDF with 10 \(\mu M\) GD1a essentially doubled the EGF-stimulated EGF receptor phosphorylation. Thus, ganglioside GD1a enhances EGF-induced EGF receptor phosphorylation.

To determine whether gangliosides affect the time course of EGF receptor activation, we exposed fibroblasts to 2 ng/ml EGF

**RESULTS**

Enhancement of EGF Receptor-mediated Signaling by Gangliosides—Ganglioside preincubation caused a dose-dependent enhancement of EGF-induced fibroblast proliferation (13). This finding points to the possibility that gangliosides enhance the EGF receptor-mediated signaling pathway. Thus, in our initial experiments we determined the effect of gangliosides on EGF receptor autophosphorylation in NHDF. We used the same conditions that we previously used (13). Following 18-h incubation of NHDF with ganglioside GD1a in FGM with 2% FBS, the cells were washed and starved in serum-free medium overnight, followed by exposure of the cells to EGF (2 ng/ml) for 5 min in serum-free medium (16).

Under these conditions, GD1a was shown to enhance EGF-induced EGF receptor phosphorylation by three experimental approaches (Fig. 1). In the first approach, the cell lysate was directly used for the detection of EGF receptor phosphorylation by an anti-phosphotyrosine antibody (Fig. 1A). Preincubation of the cells with 10 and 20 \(\mu M\) GD1a caused 28 and 56% increase in EGF-induced EGF receptor phosphorylation, respectively (Fig. 1A), whereas the total EGF receptor level was nearly equal in each sample. The EGF receptor (170 kDa) was identified by Western blot analysis using an anti-EGF receptor antibody, as well as by the standard protein marker (Fig. 1A). Additional separate experiments confirmed the enhancing effect of ganglioside GD1a on EGF receptor phosphorylation (Fig. 1C).

In the second approach, the EGF receptor phosphorylation of the same cell lysate was detected by an anti-phospho-EGF receptor antibody (Fig. 1A). GD1a preincubation clearly enhanced EGF receptor phosphorylation. Although exerting a strong enhancing effect on EGF receptor phosphorylation, ganglioside GD1a alone did not cause EGF receptor phosphorylation (Fig. 1A).
we examined the effect of G D1a on activation MAPK (p44/p42 with 10 and 20 µM Raf. In turn, Raf activates MEK and MAPK (22). As expected, that GD1a promotes fibroblast proliferation at least in part through enhancement of the EGF receptor-mediated Ras/Raf/MAPK signaling pathway.

To investigate the influence of ganglioside structure on EGF-induced signaling, we tested four highly purified gangliosides: one monosialoganglioside, G M3; two disialogangliosides, G D1a and G D3; and one trisialoganglioside, G T1b. One neutral glycosphingolipid, lactosylceramide, was also tested. As shown in Fig. 6, NHDF contain GM3 and GD3 as their major ganglioside components. GD1a and GT1b are present only in a trace amount. Ganglioside GD1a was the most active molecule in enhancing MAPK activation. On the other hand, lactosylceramide, a neutral glycosphingolipid was the least active one (Fig. 7), indicating relative structural specificity of the ganglioside effect on MAPK activation.

All the experiments above were performed under the same conditions. That is, NHDF at 70% of confluence was incubated with ganglioside G D1a in FGM with 2% FBS for 18 h, washed, and starved in serum-free medium overnight, then the cells were exposed to 2 ng/ml EGF for 5 min in serum-free medium. To investigate whether the results obtained from these experi-
iments are culture condition-dependent, we further assessed the potential influence of serum or serum starvation on the enhancing effect of GD1a on EGF receptor activation. In these experiments, cells were incubated with GD1a in either FGM with 2% FBS or in serum-free medium for 18 h, washed, and immediately exposed to EGF for 5 min without serum starvation. As shown in Fig. 8, following incubation of NHDF with ganglioside GD1a for 18 h, EGF receptor phosphorylation and MAPK phosphorylation were strikingly enhanced by ganglioside GD1a in a clear dose-dependent manner. Exposure of the cells to 10 and 20 μM GD1a caused 1- and 3-fold enhancement in EGF receptor phosphorylation, and 22 and 52% enhancement in MAPK phosphorylation. Similarly, phosphorylation of the EGF receptor and MAPK was also enhanced when NHDF were incubated with GD1a in serum-free medium for 18 h (Fig. 8). Finally, when exposed to GD1a for 6 h instead of 18 h, EGF-induced EGF receptor phosphorylation and MAPK phosphorylation were also enhanced, albeit to a lesser degree, suggesting a time-dependent ganglioside effect. For example, exposure of the cells to 20 μM GD1a in serum-free medium caused 21% increase in MAPK phosphorylation (not shown). Together, these results clearly demonstrate that exogenous addition of gangliosides under various conditions en-

FIG. 4. The enhancing effect of GD1a preincubation on EGF-induced MAPK activation. NHDF were treated, as described in Fig. 1. 20 μg of the cell lysate was subjected to SDS-PAGE and Western blot analysis, using either phospho-p44/42 MAPK or control MAPK antibodies. In addition, the MAPK activity was determined by measuring Elk-1 phosphorylation. The cell lysate containing 200 μg of total protein for each sample was immunoprecipitated using a specific anti-phospho-p44/42 MAPK antibody. The kinase assay was performed using Elk-1 as the substrate. One-third of the total product was subjected to SDS-PAGE and Western blot analysis to visualize the phosphorlated Elk-1 bands, using an anti-phospho-Elk-1 antibody. The data are the results obtained from a representative experiment. Six separate experiments were performed, and similar results were obtained.

FIG. 5. Inhibition of MAPK activation by PD98059. NHDF were seeded in six-well plates in FGM with 2% FBS and cultured until the cell density reached 70% of confluence. The cells were then cultured in FGM with 2% G_{D1a}, for 18 h, the medium was then removed, and the cells were starved in serum-free medium overnight. During the final 3 h, PD98059 (dissolved in Me_{2}SO) was added to the culture medium. After washing twice with serum-free medium, the cells were exposed to EGF (2 ng/ml) in serum-free medium for 5 min. 20 μg of the total cell lysate was subjected to SDS-PAGE and Western blot analysis, using either phospho-p44/42 MAPK or MAPK antibodies. The same lysate of cells cultured without GD1a incubation and EGF exposure was used as a negative control (NC), whereas the lysate of cells cultured with G_{D1a} and EGF (without Me_{2}SO) was used as a positive control (PC). The experiment was repeated once and similar results were obtained.

Fig. 6. HPTLC analysis of gangliosides isolated from NHDF. Total cellular gangliosides were purified from NHDF cultured in FGM with 2% FBS, and stained as purple bands on the HPTLC plate with resorcinol hydrochloric acid reagent. HBG, human brain gangliosides (4 nmol of lipid bound sialic acid) used as standard. Both G_{M3} and G_{D3} migrated as double bands on the HPTLC plate due to the ceramide heterogeneity.

Fig. 7. Influence of ganglioside structure on activity of gangliosides in enhancing MAPK phosphorylation. NHDF were seeded in six-well plates in FGM with 2% FBS. When the cell density reached 70% of confluence, the cells were incubated with 20 μM G_{M3}, G_{D1a}, G_{D3}, G_{T1b}, or lactosylceramide (Lac-cer) for 18 h. The medium was then removed, and the cells were starved in serum-free medium overnight. Finally, the cells were cultured in serum-free medium + EGF (2 ng/ml) for 5 min. 20 μg of the total cell lysate was subjected to SDS-PAGE and Western blot analysis, using either phospho-p44/42 MAPK or MAPK antibodies. The data presented are the results obtained from a typical experiment. Four separate experiments were performed, and similar results were obtained.
hances EGF receptor-mediated proliferation-coupled signaling.

Although preincubation of cells with gangliosides enhanced EGF-dependent proliferation, earlier studies in the literature show high concentrations of gangliosides inhibited cell proliferation when continuously present in the culture medium during incubation of fibroblasts with growth factors (24–31). Thus, we further evaluated the effect of the continuous presence of ganglioside GD1α in the culture medium during incubation with EGF on MAPK phosphorylation. In this experiment, GD1α was present in the culture medium throughout the assay. Consistent with our proliferation data (13), a low concentration (5 μM) caused an increase in the phosphorylation of MAPK when compared with the level of MAPK phosphorylation stimulated by EGF alone (Fig. 9). On the other hand, a high concentration of GD1α (20 μM) inhibited EGF-induced MAPK phosphorylation (Fig. 9, which is in accordance with the literature (30).

**Activation of Src Kinase by Gangliosides**—In our recent study (13), we observed that gangliosides enhanced cell proliferation in the absence of serum and growth factors. Here, we investigated how gangliosides themselves elicit proliferation signaling. Because Src kinase is located on the cytoplasmic side of the plasma membrane, and because it has been shown that Src kinase exists in glycosphingolipid-enriched domains (5, 7, 12), we determined the role of Src kinase in ganglioside-induced cell proliferation.

Following 18-h incubation of NHDF with GD1α in FGM with 2% FBS, the cells were washed and starved in serum-free medium overnight, followed by the lysis of the cells. Under these conditions, GD1α induced Src kinase phosphorylation in a dose-dependent manner (Fig. 10, A and D). Western blot analysis using an anti-Src antibody indicates that the total Src (60 kDa) in each sample was equal. Because activation of EGF receptors also activates Src kinase (1), there is the possibility that Src activation was due to the activation of EGF receptors by gangliosides. However, despite the enhancing effect on EGF-induced EGF receptor phosphorylation, GD1α alone did not cause activation of the EGF receptor (Fig. 1A). In addition, brief treatment of the cells with AG1478 (an EGF receptor inhibitor) did not block Src kinase phosphorylation induced by GD1α (Fig. 11), suggesting that GD1α activates Src kinase via another pathway.

Because of the importance of Ras in normal cell proliferation, we determined whether activation of Src by GD1α acts through a Ras-dependent pathway. As shown in Fig. 3B, in the absence of EGF, GD1α exposure clearly activated Ras. Whereas the total Ras remained at the same level, 20 μM GD1α caused a striking increase (119%) in the level of Ras-GTP, which is nearly equivalent to the effect of 2 ng/ml EGF (Figs. 3, B and D).

GD1α preincubation also activated MAPK in a dose-dependent manner. As shown in Fig. 10B, exposure of the cells to 10, 20, and 50 μM GD1α induced 27-, 39-, and 74-fold stimulation in MAPK phosphorylation, respectively. In this experiment, 50 μM GD1α had an effect equivalent to ~80% of the effect of 2 ng/ml EGF (Fig. 10B). The MAPK phosphorylation, in turn, resulted in Elk-1 phosphorylation (Fig. 10C). For example, exposure of the cells to 20 and 50 μM GD1α induced 10- and 23-fold stimulation in Elk-1 phosphorylation. Pretreatment of the cells with PD98059 for 3 h effectively blocked MAPK activation (Fig. 12). These results suggest that gangliosides, in the absence of serum, activate the Src→Ras→MEK→MAPK pathway.

To determine whether the structure of gangliosides influences the activation of Src kinase and MAPK, we tested four highly purified gangliosides: G3F3, GD3, and GT1b. Lactosylceramide, a neutral glycosphingolipid, was also tested. At 20 μM, all four gangliosides were active in stimulating Src kinase phosphorylation, with G3F3 and GD3 being the most active molecules (Fig. 13A). Lactosylceramide, on the other hand, was inactive, suggesting that the intact ganglioside structure is important in activating Src kinase. In accordance with these observations, GD1α, GM3, and GT1b were also active in enhancing MAPK activation, whereas lactosylceramide was not active (Fig. 13B), again indicating the relative specificity of the ganglioside effect.

In the above experiments to assess the ganglioside effect on
Src kinase and MAPK activation by GD1a. NHDF were seeded at 2 × 10⁵ cells in six-well plates in FGM with 2% FBS and cultured until they reached 70% of confluence. The cells were then cultured in FGM with 2% FBS until their density reached ~70% of confluence. The cells were washed and cultured in serum-free medium overnight, followed by the lysis of the cells to determine Src (A) and MAPK activation (B and C). 20 μg of the total cell lysate was subjected to SDS-PAGE and Western blot analysis, using either a phospho-Src kinase antibody or a c-Src antibody (A). For determination of MAPK phosphorylation status (B), 20 μg of the total cell lysate was subjected to SDS-PAGE and Western blot analysis, using either phospho-p44/42 MAPK or MAPK antibodies. The MAPK activity was determined by measuring Elk-1 phosphorylation (C). The cell lysate containing 200 μg of total protein for each sample was immunoprecipitated using a specific anti-phospho-p44/42 MAPK antibody. The kinase assay was performed using Elk-1 as the substrate. One-third of the total reaction product was subjected to SDS-PAGE and Western blot analysis to visualize the phosphorylated Elk-1 bands, using anti-phospho-Elk-1 antibody. The lysate of cells stimulated by EGF was used as a positive control. A phosphorylated Elk-1 protein was used as a standard. The data presented in each panel are the results obtained from a typical experiment. Five separate experiments for A, four separate experiments for B, and two separate experiments for C were performed, respectively, and similar results were obtained. D, relative optical intensity of the phosphorylated Src bands from Western blot analysis (A, top). The results represent the mean ± S.D. of densitometric scanning of four gels from four separate experiments.

Src kinase activation, NHDF were incubated with gangliosides in FGM with 2% FBS for 18 h, washed, and starved in serum-free medium overnight. To investigate whether the induction of Src kinase activation by gangliosides is culture condition-dependent, we examined the potential influence of serum or serum starvation on ganglioside-induced Src kinase activation. In these experiments, NHDF were incubated with ganglioside GD1α in either FGM with 2% FBS or in serum-free FBM for 18 h, washed, and immediately lysed. As shown in Fig. 14, whether serum (2% FBS) was present or not during the incubation of cells with GD1α, phosphorylation of Src kinase and MAPK was strikingly enhanced in a clear dose-dependent manner. For example, when NHDF were incubated with GD1α in serum-free medium for 18 h and without serum starvation, 10 and 20 μM GD1α induced an 11- and 12-fold increase in phosphorylation of Src kinase, and a 33- and 68-fold increase in phosphorylation of MAPK, respectively. The stimulation of Src and MAPK by GD1α was independent of the specific assay procedures used. Together, these results demonstrate that exogenous addition of gangliosides in the culture medium induces proliferation-coupled signaling via Src kinase and MAPK activation.

The prolonged activation of Src kinase caused by ganglioside treatment is unusual. Thus, we further investigated the activation of Src kinase using three approaches: an Src kinase inhibitor, measurement of plasma membrane-bound Csk, and assessment of Src kinase activity. When PP1, a Src kinase inhibitor (15), was added to the culture medium during the final 3 h of the ganglioside incubation, phosphorylation of both Src kinase and MAPK (Fig. 15) was clearly reduced at 1 μM PP1 and abolished at 50 μM PP1. The inhibition of Src kinase by PP1 confirmed that gangliosides act to stimulate Src kinase.

It is known that when Src is activated, a Csk-binding protein, which is anchored in the membrane, binds to Csk and...
brings Csk to the membrane from the cytosol. The recruited Csk then adds an inhibitory phosphate to Src kinase and removes an activating phosphate (5). To investigate whether ganglioside incubation results in any changes in the level of Csk, we determined the Csk level in total cell lysate and plasma membrane. Following 18-h incubation with 0–50 μM H9262M GD1a and overnight serum starvation, the level of total cellular Csk remained constant, whereas the plasma membrane-bound Csk level increased (Fig. 16).

To confirm the enhancement of Src kinase phosphorylation detected by Western blot analysis, we evaluated Src kinase activity by measuring the phosphorylation of a substrate peptide. NHDF were cultured in FGM containing 2% FBS medium and 0–50 μM H9262M GD1a for 18 h and starved in serum-free medium overnight, followed by the lysis of the cells to determine Src and MAPK phosphorylation. The data presented in each panel are the results obtained from a typical experiment. Three separate experiments were performed, and similar results were obtained.

Finally, we determined the time course of Src activation in response to EGF and GD1a in four parallel experiments. In the first experiment (Fig. 18A), NHDF were preincubated with 20 μM GD1a in FGM with 2% FBS for 18 h, starved in serum-free medium for 18 h, and stimulated with 2 ng/ml EGF for 5 min. The cells were then washed and further cultured in serum-free medium for up to 6 h, during which the Src activity was measured. As expected, stimulation of the cells with EGF rapidly activated Src kinase. Within 5 min, Src activity reached the maximal level, and after 30 min, it had returned to the basal level (Fig. 18A). In the second experiment (Fig. 18B), the experimental approach was the same as that of the first experiment, except NHDF was stimulated with a second dose of 20 μM GD1a for 1 h instead of being stimulated by EGF. As shown in Fig. 18B, Src activity was highly elevated (indicated by "a") following GD1a preincubation and 18-h starvation. Upon stimulation by a second dose of GD1a, Src activity further increased to a higher level (indicated by "b"). Src remained activated at the higher level over the 6-h period of serum-free culture. In the third experiment (Fig. 18C), NHDF were incubated with 20 μM GD1a in FGM with 2% FBS for 18 h, followed by incubation of the cells with 20 μM GD1a in serum-free medium for 18 h. The cells were then washed and cultured in serum-free medium for up to 6 h, during which Src activity was measured. The prolonged incubation of NHDF with GD1a resulted in an increase in Src activity in a time-dependent manner (Fig. 18C). Similarly, 6-h incubation of the cells with 20 μM GD1a in serum-free medium in experiment 4 also caused a time-dependent increase.
DISCUSSION

Our recent study has delineated a stimulatory role of membrane gangliosides in fibroblast proliferation (13). Inhibition of cellular ganglioside synthesis blocked growth factor-induced fibroblast proliferation. Conversely, enrichment of cell membrane gangliosides by ganglioside preincubation enhanced growth factor-elicited cell proliferation. In the absence of serum and growth factors, GD1a acted like a growth factor when cells were pretreated with the ganglioside, stimulating proliferation of fibroblasts. Here, we demonstrate two independent mechanisms whereby gangliosides elicit proliferation-coupled signaling in normal human dermal fibroblasts. First, gangliosides enhance EGF receptor-mediated signaling. Incubation of the fibroblasts with GD1a enhanced EGF receptor autophosphorylation and Ras/MAPK activation in a dose-dependent manner. Although enhancing EGF receptor phosphorylation, GD1a itself did not cause EGF receptor phosphorylation. Pretreatment of the cells with PD98059 blocked the enhancement of gangliosides on EGF-induced MAPK activation. Second, gangliosides stimulate Src kinase activity. In the absence of serum and growth factors, ganglioside GD1a incubation induced the phosphorylation of Src kinase, Ras activation, and phosphorylation of MAPK in a dose-dependent manner. Brief treatment of the cells with PP1 blocked the activation of Src kinase and MAPK. Again, PD98059 treatment inhibited ganglioside-elicited MAPK phosphorylation. Thus, gangliosides enhance growth factor signaling and activate Src kinase. In addition, the apparent paradox regarding the effect of gangliosides on cell proliferation was resolved in this study. That is, consistent with our proliferation data (13), when continuously present in the culture medium during incubation of fibroblasts with EGF, a low concentration of GD1a (5 μM) caused an increase in the phosphorylation of MAPK. In contrast, when continuously present in the culture medium during incubation of fibroblasts with EGF, a high concentration of GD1a (50 μM) inhibited EGF-induced MAPK phosphorylation. This observation is consistent with the literature that high concentrations of gangliosides inhibited cell growth when continuously present in the culture medium during incubation of cells with growth factors (24–31).

A study by Saqr et al. (32) reports that GM1, GD1a, and GT1b stimulate DNA synthesis in human glioma U-1242 cells and the ganglioside effect on DNA synthesis was more prominent in quiescent, confluent cells than in quiescent, sparse cells. The
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authors suggest that the exogenous ganglioside effect depends on state of confluence (cell contact) and that gangliosides have a bimodal effect on DNA synthesis (32).

How do gangliosides enhance EGF receptor-mediated signaling and stimulate non-receptor Src kinase activity? The ceramide portion of gangliosides anchors the molecules in the plasma membrane, and gangliosides themselves are generally believed to be devoid of enzymatic features. Hence, gangliosides transduce signals possibly by association with growth factor receptors. Presumably, aggregation of growth factor receptors with gangliosides in the membrane domains brings the growth factor signaling to a threshold of activity and optimal signaling and thus promotes receptor dimerization and phosphorylation, leading to enhanced MAPK activation and cell proliferation. Although this study focused on EGF receptors, the conclusion here is likely applicable to other proliferation-coupled receptor tyrosine kinases, such as the platelet-derived growth factor receptor and fibroblast growth factor receptor. How can gangliosides stimulate Src kinase? One possibility is that gangliosides inserted in the plasma membrane interact with the non-receptor Src tyrosine kinase that is located in the inner leaflet via N-terminal lipid modification (palmitoylation and myristoylation). Another possibility is that ganglioside molecules inserted in the plasma membrane interact with Src kinase negative regulators, removing inhibition of Src kinase and consequently causing activation of Src kinase (5). In Neuro2a cells, the Csk/c-Src ratio was decreased in glycosphingolipid-enriched domains following G_{34} treatment (12). What is found here is an increase in the level of plasma membrane-bound Csk. Why ganglioside incubation increased membrane Csk and how such an increase correlates with the prolonged activation of Src kinase are unknown. Future studies are needed to address these issues.

Lipid microdomains have been implicated in signal transduction. Glycosphingolipids, sphingomyelin, and cholesterol cluster together to form lipid microdomains within the fluid lipid bilayer (6, 7, 33, 34). Glycosphosphatidylinositol-anchored proteins and other signaling molecules are enriched in these microdomains (35). Upon cross-linking, B cell receptors rapidly translocate into ganglioside G_{34}-enriched domains (36, 37). T cell receptor cross-linking causes aggregation of raft-associated proteins, which in turn promotes tyrosine phosphorylation and signaling protein recruitment (38). Targeting of T cell receptor signaling molecules to glycosphingolipid-enriched microdomains is considered critical for T cell activation (39). Incubation of the cells with gangliosides causes insertion of the molecules in the plasma membrane (40, 41), conceivably enhances the formation of lipid microdomains, and promotes EGF-receptor mediated signaling, as observed in the present study. Conversely, depletion or reduction of membrane gangliosides (13, 42) may adversely affect the ability of EGF receptor to properly localize to lipid microdomains before stimulation or to exit the microdomains upon stimulation with EGF.

Recently, the concept of “glycosignaling domains” has been proposed, to emphasize that clustered glycosphingolipidstems themselves may initiate signal transduction through the interaction of their lipid portion with aliphatic chains of transducer molecules (7, 10). Association of Src family tyrosine kinase Lyn with ganglioside G_{34} was reported in rat brain (43). Subsequently, the glycosphingolipid-containing membrane domains isolated from mouse melanoma B16 cells (7) and mouse neuroblastoma Neuro2a cells (12) were found to contain c-Src. In Neuro2a cells that are responsive to gangliosides but not to nerve growth factor, addition of G_{34} to the cells caused enhanced c-Src phosphorylation and MAPK activation leading to neuritogenesis. Brief stimulation of isolated glycosphingolipid-enriched domains by G_{34} also resulted in enhanced c-Src phosphorylation, suggesting that ganglioside induction of neuritogenesis in Neuro2a cells is mediated by glycosphingolipid-enriched domains (12). In the present study, ganglioside G_{34} and other gangliosides, G_{33}, G_{3}, and G_{T}b, were found to activate Src, Ras, and MAPK, leading to enhanced fibroblast proliferation (13). These findings clearly provide evidence on the role of gangliosides in proliferation-coupled signal transduction.

In conclusion, gangliosides elicit proliferation-coupled signaling through enhancement of growth factor signaling and activation of Src kinase. The delineation of ganglioside-mediated signaling may provide a new approach to regulate cell proliferation. Further studies are needed to determine how gangliosides interact with the growth factor receptor in the plasma membrane and how gangliosides activate Src kinase, with the goal of elucidating the biological functions of gangliosides in cell proliferation and signal transduction.

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