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In Vivo Modulation of Epidermal Growth Factor Receptor Phosphorylation in Mice Expressing Different Gangliosides

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Abstract We studied in this work the in vivo phosphorylation of the epidermal growth factor receptor (EGFr) in skin from knockout mice lacking different ganglioside glycosyltransferases. Results show an enhancement of EGFr phosphorylation, after EGF stimulation, in skin from Sial-T2 knockout and Sial-T2/GalNAc-T double knockout mice as compared with wild-type and Sial-T1 knockout mice. Qualitative analysis of ganglioside composition in mice skin suggest that the increase of EGFr phosphorylation observed in skin from Sial-T2 knockout and Sial-T2/GalNAc-T double knockout mice in response to EGF might not be primary attributed to the expression of GD3 or *a*-series gangliosides in mice skin. These studies provide, for the first time, an approach for studying the molecular mechanisms involved in the in vivo regulation of EGFr function by gangliosides. J. Cell. Biochem. 99: 1442–1451, 2006.

Key words: gangliosides; EGF receptor; ganglioside glycosyltransferase; skin; mice; glycolipid

Gangliosides, glycosphingolipids containing sialic acid, have been found in almost all mammalian plasma membranes where they participate in recognition and signaling activity. A combinatorial biosynthetic pathway in Golgi complex results in a large diversity of oligosaccharide structures on gangliosides [Kolter et al., 2002] (Fig. 1). On the basis that the bulk of gangliosides present in the cell are plasma membrane bound and projecting their oligossacharide cores toward the extracellular space, it has been speculated that they participate in cell-surface events such as phosphorylation of tyrosine kinase growth factor receptors. In this sense, it has been demonstrated, mainly by exogenous addition of gangliosides or changes of the endogenous contents by using molecular biology, that gangliosides regulate the activity of TrkA receptor [Mutoh et al., 1995; Fukumoto et al., 2000], insulin receptor [Allende and Proia, 2002; Yamashita et al., 2003], epidermal growth factor receptor (EGFr) [Miljan and Bremer, 2002; Liu et al., 2004], and platelet derived growth factor receptor [Yates et al., 1995; Mitsuda et al., 2002].

EGFr is a large transmembrane glycoprotein (170 kDa) with ligand-inducible tyrosine kinase activity. Binding epidermal growth factor (EGF) to EGFr triggers the phosphorylation of tyrosine residues and initiates a kinase cascade

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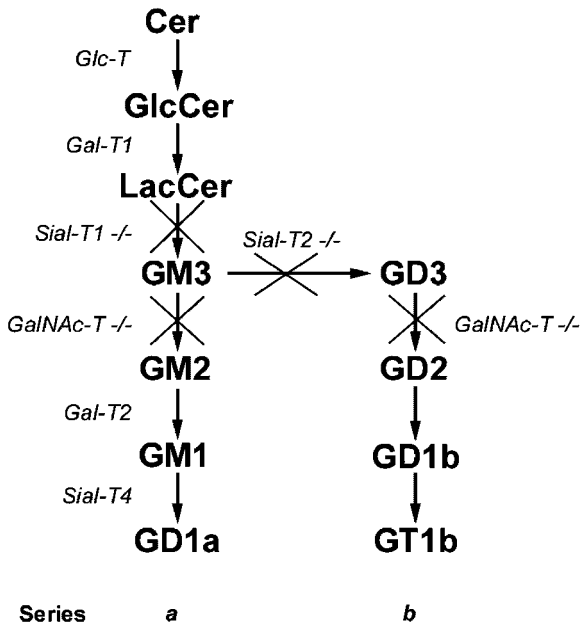

Fig. 1. Pathway of biosynthesis of *a*- and *b*-series gangliosides. The blockage of ganglioside biosynthesis by the disruption of the genes, *Sial-T1*, *Sial-T2*, and *GalNAc-T*, is indicated. Cer, ceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide.

that results in the activation of mitogen-activated protein kinase (MAPK). An intermediate in this cascade is the GTP-binding protein Ras. Ras controls the recruitment of RAF-1 to the plasma membrane after EGF binding where it is activated by phosphorylation and becomes available to phosphorylate MAPK kinase [Moghal and Sternberg, 1999].

We have previously described the modulation of EGFr phosphorylation by endogenously expressed gangliosides [Zurita et al., 2001, 2004]. Particularly, we observed an inhibition of EGFr autophosphorylation when Chinese hamster ovary (CHO)-K1 cells ($GM3^+$) were induced to express the disialoganglioside GD3 (gangliosides are named according to [Svennerholm, 1963]) by stable transfection of CMP-sialic acid:GM3 α2,8-sialyltransferase (Sial-T2, GD3 synthase). To determine the physiological role of both GD3 and other gangliosides on EGFr activity, we studied in this work the in vivo phosphorylation of the receptor, after EGF stimulation, in skin from knockout mice for CMP-sialic acid:lactosylceramide α2,3-sialyltransferase (Sial-T1, GM3 synthase), Sial-T2 and double mutant mice for Sial-T2 and UDP-N-acetyl-D-galactosamine:GM3/GD3 *N*-acetyl-D-galactosaminyltransferase (GalNAc-T, GM2/GD2 synthase) (Fig. 1). Our results show a higher enhancement of EGFr phosphorylation, after EGF stimulation, in skin from Sial-T2 knockout and Sial-T2/GalNAc-T double knockout mice than in wild-type and Sial-T1 knockout mice. The analysis of ganglioside composition in skin from normal and mutant mice strongly suggest that GD3 or *a*-series gangliosides (GM3, GM1, and GD1a) might not be a primary factor to modulate EGFr phosphorylation in response to EGF in mice skin.

MATERIALS AND METHODS

Mutant Mice

The following mice, lacking different ganglioside glycosyltransferases, were used: Sial-$T1^{-/-}$ mice, these mice lack CMP-sialic acid:lactosylceramide α2,3-sialyltransferase; EC 2.4.99.−; GM3 synthase [Yamashita et al., 2003]; Sial-$T2^{-/-}$ mice, these mice lack CMP-sialic acid: GM3 α2,8-sialyltransferase; EC 2.4.99.8; GD3 synthase [Kawai et al., 2001]; Sial-$T2^{-/-}$ and GalNAc-$T^{-/-}$ mice, these double mutant mice lack Sial-T2 and UDP-*N*-acetyl-D-galactosamine: GM3/GD3 *N*-acetyl-D-galactosaminyltransferase; EC 2.4.1.92; GM2/GD2 synthase [Kawai et al., 2001]. The blockage of ganglioside biosynthesis by the disruption of the genes, Sial-T1, Sial-T2, and GalNAc-T is shown in Figure 1. All animal procedures were approved by the National Institute of Diabetes and Digestive and Kidney Diseases and were performed in accordance with the National Institutes of Health guidelines.

In Vivo Experiments

Solutions of EGF (1 mg/ml, Gibco-BRL) in PBS were injected subcutaneously into neonatal mice (1-day-old) at a dose of 10 μl/g of body weight. Control animals received injections of PBS. Mice were kept on a warm pad at 37°C during the EGF stimulation. Ten minutes later, mice were sacrificed and organs of interest were excised immediately. Tissues were homogenized (3 ml of ice-could buffer per gram of tissue) on ice with a dounce homogenizer in a solution of PBS containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 10 μg/ml aprotinin, and 10 μg/ml leupeptin [Donaldson and Cohen, 1992]. Homogenates were incubated on ice for 30 min and then centrifuged at 15,000*g* for

20 min at 4°C. Aliquots of the supernatants were processed for electrophoresis and Western blot.

For ex vivo experiments, skin biopsies were prepared from wild-type mice, which were incubated at 37°C in an eppendorff containing 50 μl of a solution without or with EGF (1 mg/ml) in PBS at the indicated time. Homogenates from these samples and containing equal amount of protein were run in SDS–PAGE and processed for Western blot as described below (Electrophoresis and Western Blot).

Electrophoresis and Western Blot

Electrophoresis and transfer was carried out as previously described [Zurita et al., 2001]. Membranes were blocked with 5% non-fat dry milk or with 2.5% bovine serum albumin (BSA)-2.5% polyvinyl pyrrolidone 40 in Tris-buffered saline (200 mM NaCl, 100 mM Tris-HCl, pH 7.5), depending on the antibody. Antibodies antiphosphotyrosine (PY20:*sc-508*, Santa Cruz Biotechnology), antiphospho-MAPK (#9106S, Cell Signaling Technology), antiMAPK (#9102, Cell Signaling Technology), and antiEGFr (#E12020 from Transduction Laboratories; EGFR (1005):*sc-03* and EGFR (1005):*sc-03*-G from Santa Cruz Biotechnology) were used at a dilution of 1:1,000, respectively. sc-03 (rabbit IgG), sc-03-G (goat IgG), and #E12020 (mouse IgG1) were raised against a peptide mapping at the carboxy terminus of the EGF receptor of human origin. Bands were detected by protein A or appropriate secondary antibodies coupled to horseradish peroxidase combined with the chemiluminescence detection kit (Western lightning, PerkinElmer Life Sciences, MA) and Kodak Biomax MS films. The molecular weights were calculated based on calibrated standards (Gibco-BRL) run in every gel. The relative contribution of individual bands was calculated using the computer software Scion Image on scanned films of low-exposure images. MAPK level was used to normalize level of proteins seeded in each lane. For sequential Western blot using the same membrane, antibodies were removed by incubation of the nitrocellulose membrane in NaOH 1N during 5 min. After a proper blocking, membranes were incubated with the corresponding antibody.

Immunoprecipitation Assay

Homogenates from control and EGF-treated skin were prepared as described above (In Vivo Experiments). Then, lysates were centrifugated at 400*g* for 10 min and the supernatants mixed with protein A-Sepharose beads and precleared by stirring for 30 min at 4°C. Then, supernatants were incubated with antiEGFr antibody and protein A-Sepharose at 4°C during 4 h. After a brief centrifugation, the supernatants were discarded and beads were washed three times with PBS buffer containing 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and appropriated inhibitors of proteases and phosphatases. The immunoprecipitates were separated by SDS–PAGE, and analyzed by Western blot using antiphosphotyrosine and antiEGFr antibodies.

Glycolipids Extraction and Chromatography

Briefly, lipids from skin tissue were first extracted with chloroform:methanol (2:1 v/v) during 12 h and then re-extracted with chloroform:methanol:H_2O (30:60:8) during 12 h. Then, lipid extracts were combined and subjected to Folch partition [Folch et al., 1957]. The resulting aqueous phases were freed from salt and other contaminants by passing through Sep-Pak C_{18} cartridge column (Waters Corporation Milford). The eluted glycolipid fraction was chromatographed on high-performance thin layer chromatography plates (HPTLC, Merck, Germany) using chloroform:methanol:0.2% CaCl2 (60:36:8 v/v) as solvent. Standard cochromatographed gangliosides and gangliosides from skin tissue were visualized by exposure of the plate to iodine vapors and by staining with orcinol reagent [Crespo et al., 2004]. The relative contribution of individual bands was calculated using the computer software Scion Image on scanned films.

Immunological Detection of GD3 on the Polyvinylidene Difluoride Membrane

Glycosphingolipids on the developed HPTLC plate were blotted to a polyvinylidene difluoride (PVDP) membrane as previously reported [Taki et al., 1994]. After blotting, the PVDF membrane was dried, immersed in 1% BSA in PBS to block nonspecific protein binding, and incubated at 4°C for overnight in a nylon bag containing the monoclonal antibody R24 (mouse IgG3) that recognize the ganglioside GD3 [Pukel et al., 1982]. The membrane was rinsed in PBS and then incubated with the second antibody, peroxidase-conjugated antimouse

IgG (1:5,000 dilution in Tris-buffered saline containing 0.05% Tween 20). After incubation at room temperature for 2 h, the membrane was again rinsed in PBS, and the GD3 on it was detected using the chemiluminescence detection kit (Western lightning, PerkinElmer Life Sciences).

RESULTS

In Vivo EGFr Stimulation

EGFr are much more abundant in the rapidly growing epidermis of neonatal mice, undergoing a strong downregulation a few days after birth [Green et al., 1983]. Therefore, EGF in PBS was subcutaneously administrated to wild-type neonatal mice (1-day-old). Control animals received injections of PBS. After 10 min, skin and other organs such as kidney, brain, and lung were excised and processed for Western blot analysis using antiphosphotyrosine antibody. The results are illustrated in Figure 2A. Clearly, the administration of EGF resulted in an enhancement of tyrosine phosphorylation in proteins from skin with an apparent molecular mass of ~170 and ~100 kDa. Next, antibodies were removed and the same nitrocellulose membrane was used to evaluate the expression level of total EGFr in the different tissues. A high expression of EGFr was observed in skin. However, when this tissue was incubated with EGF, a reduction in the detection of total EGFr was observed. This decrease in EGFr could be explained by the rapid receptor downregulation, which takes place upon receptor activation, or by a reduced immunoreactivity of the antiEGFr for the phosphorylated receptor. To further elucidate the apparent decrease in total EGFr after stimulation, we performed a time course experiment after EGF administration (Fig. 2B). Basically, skin biopsies were prepared from wild-type mice, which were incubated with EGF (1 mg/ml) at the indicated time. The results clearly showed the inverse correlation between the immunoreactivity of the antiEGFr with the degree of EGFr phosphorylation and, as a consequence, with its capacity to activate the MAPK signal cascade.

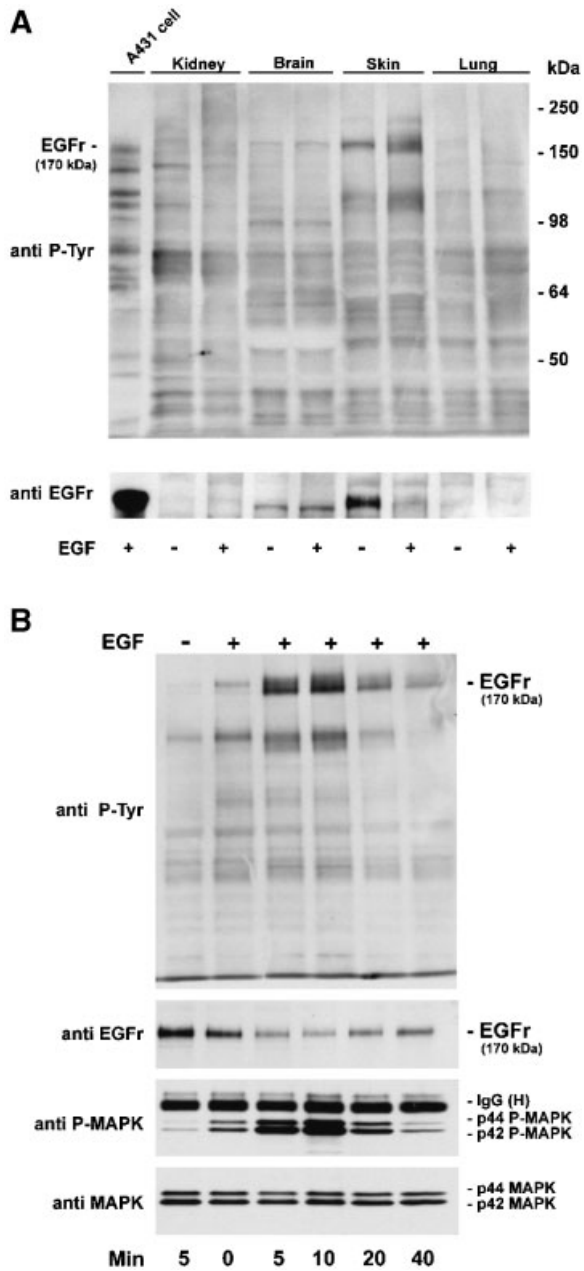

Fig. 2. In vivo EGFr stimulation. **A**: EGF was subcutaneously administrated to wild-type neonatal mice. After 10 min, skin, kidney, brain, and lung were excised and processed for Western blot analysis using antiphosphotyrosine antibody (antiP-Tyr, upper panel). Next, the same membrane was processed for Western blot analysis using antitotal EGFr (antiEGFr, lower panel). Sizes of markers in kDa are indicated. An extract from EGF-stimulated A431 cells was used as control (A431 cells). **B**: Time course of EGFr phosphorylation after EGF stimulation. Skin biopsies were prepared from wild-type mice and incubated with EGF at the indicated time (Min, minute). The phosphotyrosine pattern was analyzed by Western blot (antiP-Tyr, upper panel). Then, the membrane was Western blotted with antiphospho MAPK (antiP-MAPK, active form), which recognizes the active form of MAPK. Next, the membrane was processed for Western blot analysis using antitotal MAPK (antiMAPK, lower panel), and antitotal EGFr (middle panel). The positions of p42 and p44 MAPK, the heavy (H) chain of IgG immunoglobulin and EGFr (170 kDa) are indicated.

To further demonstrate that the band of ~170 kDa observed in skin corresponded to EGFr, extracts from control and EGF-treated skin were incubated with an antibody anti-EGFr, and the antigen–antibody complexes were isolated using protein A-Sepharose. Then, the complexes were separated by SDS–PAGE, and analyzed by Western blot using antiphosphotyrosine antibody (Fig. 3A). Results indicate that the band of 170 kDa observed in skin with the antiphosphotyrosine antibody corresponds to the EGFr protein. As also shown in Figure 2B, a clear coupling and activation of MAPK (p42 and p44 MAPK) detected with an antiphopho-MAPK (active form) was also observed in skin after EGF stimulation (Fig. 3B). Taken all these experiments together, results indicate that binding of EGF to EGFr expressed in skin-activated phosphorylation of the receptor and generated a stimulus able to couple with and activate MAPK, an intermediate in the EGF signal cascade.

EGFr Phosphorylation in Skin From Knockout Mice of Different Ganglioside Glycosyltransferases

Having shown and characterized that EGFr from skin can be activated in vivo after EGF stimulation; we decided to analyze the phosphorylation of the EGFr in the diverse glycolipid environment provided by the membranes from different ganglioside glycosyltransferases knockout mice. Using essentially the approach described above, we examined the phosphorylation of EGFr, after EGF stimulation, in skin from wild-type, Sial-T1$^{-/-}$; Sial-T2$^{-/-}$, and Sial-T2$^{-/-}$ and GalNAc-T$^{-/-}$ (double knockout) mice. Figure 1 shows the biosynthetic pathway of gangliosides and the blockage of ganglioside synthesis by the disruption of glycosyltransferase genes. Figure 4A,B shows two typical experiments carried out in different knockout mice in which each lane corresponds to individual animals. EGFr phosphorylation, at 10 min

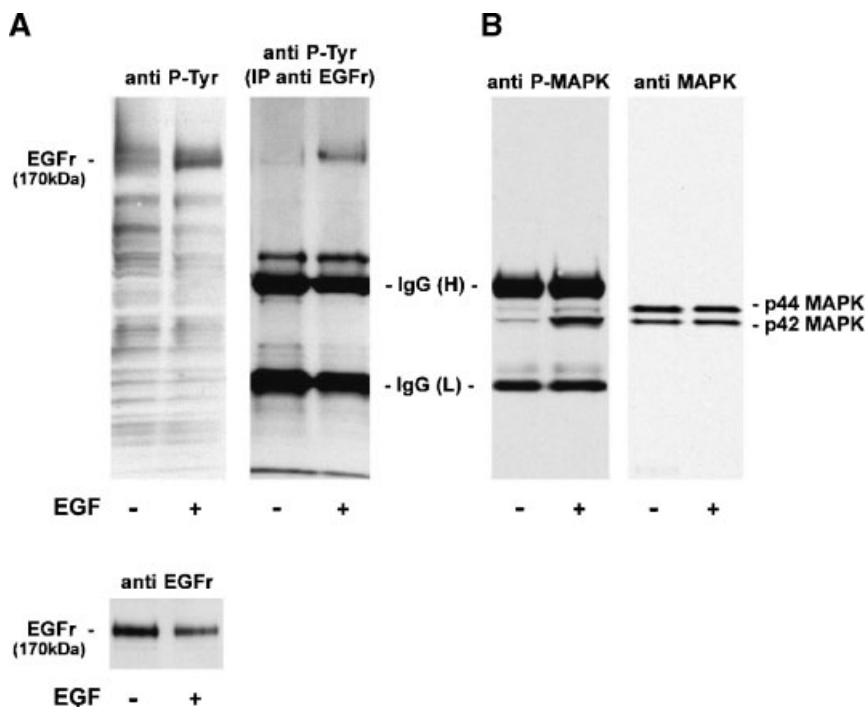

Fig. 3. Identification of the tyrosine-phosphorylated 170 kDa band as the receptor for EGF. **A**: Extracts from control (−) and EGF-treated (+) skin were processed for Western blot analysis using antiphosphotyrosine antibody (antiP-Tyr, left panel). Total EGFr expression in mice skin was evaluated by Western blot with an antibody developed in goat (antiEGFr). Extracts from control and EGF-treated skin were also incubated with antiEGFr antibody and the antigen–antibody complexes isolated using protein A-Sepharose. The immunoprecipitates (IP) were separated by SDS–PAGE, and analyzed by Western blot using antiphosphotyrosine (antiP-Tyr). **B**: In another experiment, extracts from control and EGF-treated skin were processed for Western blot analysis using antiphospho MAPK (antiP-MAPK). Next, antibodies were removed and the same nitrocellulose membrane was processed for Western blot analysis using antitotal MAPK (antiMAPK, right panels). The positions of p42 and p44 MAPK, the heavy (H) and light (L) chains of IgG immunoglobulin and EGFr are indicated.

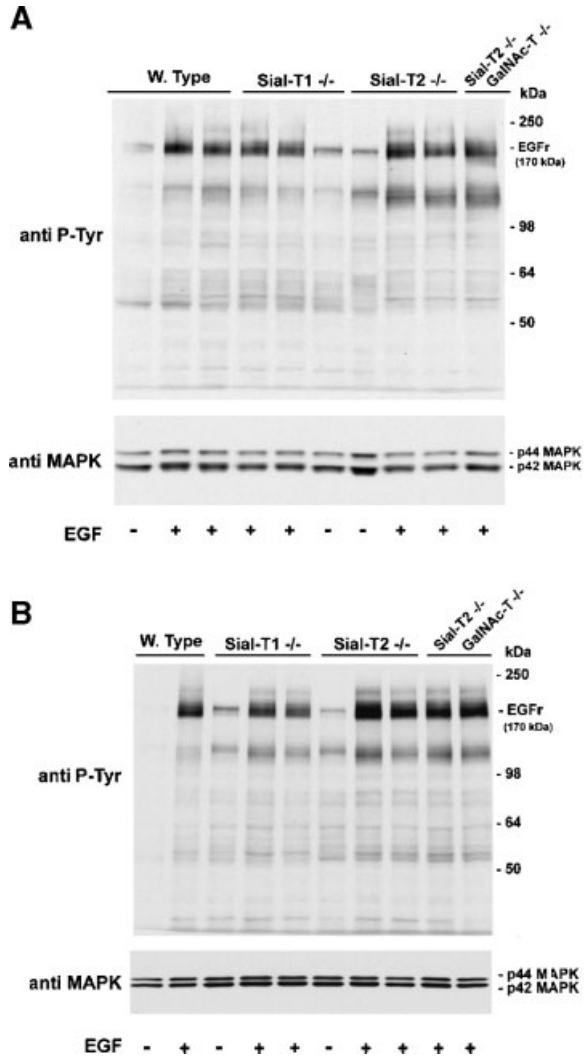

Fig. 4. EGFr phosphorylation in skin from knockout mice of different ganglioside glycosyltransferases. **A**: EGF in PBS was subcutaneously administrated to wild-type, Sial-T1$^{-/-}$; Sial-T2$^{-/-}$, and Sial-T2$^{-/-}$ and GalNAc-T$^{-/-}$ double knockout neonatal mice. Control animals received injections of PBS. After 10 min, skin was excised and processed for Western blot analysis using antiphosphotyrosine antibody (antiP-Tyr, upper panel). Next, antibodies were removed and the same nitrocellulose membrane was processed for Western blot analysis using antitotal MAPK (antiMAPK, lower panel). **B**: Same experiment as in A. The positions of p42 and p44 MAPK and EGFr are indicated. Sizes of markers in kDa are also indicated. Each lane in A and B corresponds to individual animals.

after injection of EGF, was essentially comparable both in wild-type and in Sial-T1$^{-/-}$ mice. In contrast, in Sial-T2$^{-/-}$ mice the phosphorylation of EGFr noticeable and reproducible increased after EGF stimulation in comparison with the other mice. Moreover, the higher enhancement of EGFr phosphorylation in Sial-T2$^{-/-}$ mice was independent of GalNAc-T gene expression because almost the same result was obtained in Sial-T2$^{-/-}$ and GalNAc-T$^{-/-}$ double knockout mice. At first interpretation and coupled with the analysis of ganglioside expression in mice skin (described below) these results suggest that GD3, GM3 alone or GM3 plus α-series gangliosides might not be involved in the higher enhancement of EGFr phosphorylation in response to EGF in mice skin. As control of protein loading, we analyzed the expression of MAPK. No substantial differences were observed.

As a further evaluation of phosphorylated-EGFr level in the different conditions, quantification of the signal was performed by densitometric analysis (Fig. 5). The results obtained confirmed our qualitative observation. EGFr phosphorylations in skin from Sial-T2$^{-/-}$ mice increased to approximation 180% of that of the other mice. We have also taken into consideration the possibility that the increase of the phosphorylated-EGFr can be due to an increase of the protein content both by enhanced synthesis or reduced degradation. We quantified EGFr expression in the different mutant mice by Western blot with the antibody raised in rabbit, which showed a reduced dependency on the status of EGFr phosphorylation. After densitometric analysis of bands, a ratio between phosphorylated-EGFr and total EGFr was done. Results were essentially the same showed in Figure 5 and support the notion that there is an increase of EGFr phosphorylation instead an increase of EGFr protein content (Fig. 6).

Ganglioside Expression in Mice Skin

We performed a qualitative and quantitative analysis of gangliosides in skin from normal newborn (1-day-old) and adult mice. We observed that gangliosides from the α-series pathway were predominantly expressed at both stages (Fig. 7A). GM3 (25%), GM1 (30%), and GD1a (45%) were found to be the major gangliosides expressed in 1-day-old skin while GM3 (40%) and GM1 (31%) were the main gangliosides expressed at adult stage, with a slight reduction in the relative expression of GD1a (29%) compared to 1-day-old skin. An additional faint band running as the GD3 standard was mainly observed at the adult stage (Fig. 7A). However, we could not detect GD3 ganglioside in adult skin by using highly sensitive and specific immunological procedures such as HPTLC blotting (Fig. 7B) and immunocytochemistry in

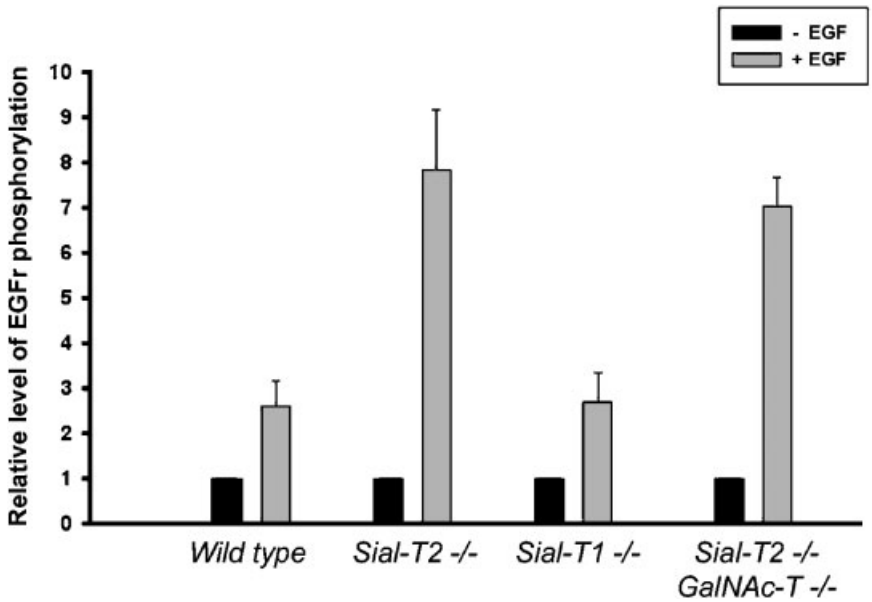

Fig. 5. Quantification of EGFr phosphorylation in skin from knockout mice of different ganglioside glycosyltransferases. EGF in PBS was subcutaneously administrated to wild-type, Sial-T1$^{-/-}$, Sial-T2$^{-/-}$, and Sial-T2$^{-/-}$ and GalNAc-T$^{-/-}$ double knockout neonatal mice (+EGF). Control animals received injections of PBS (-EGF). After 10 min, skin was excised and processed for Western blot analysis using antiphosphotyrosine antibody. Next, antibodies were removed and the same nitrocellulose membrane was processed for Western blot analysis using antitotal MAPK. The relative contribution of phosphorylated-EGFr bands was calculated using the computer software Scion Image on scanned films of low-exposure images. The intensity of the phosphorylated-EGFr band in control mice (-EGF) running at 170 kDa in SDS–PAGE was arbitrarily taken as 1. MAPK level was used to normalize level of proteins seeded in each lane. Values for phosphorylated-EGFr in EGF stimulated mice are shown as mean ± SD.

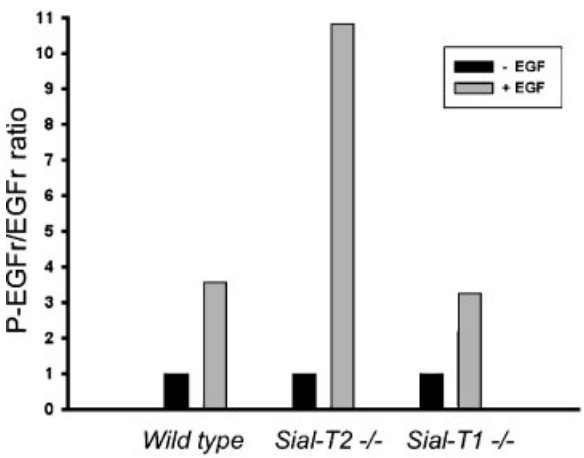

Fig. 6. Quantification of phosphorylated-EGFr (P-EGFr) in skin from knock out mice of different ganglioside glycosyltransferases. EGF in PBS was subcutaneously administrated to wild-type, Sial-T1 $^{-/-}$ and Sial-T2 $^{-/-}$ neonatal mice (+EGF). Control animals received injections of PBS (-EGF). After 10 min, skin was excised and processed for Western blot analysis using antiphosphotyrosine antibody. Next, antibodies were removed and the same nitrocellulose membrane was processed for Western blot analysis using antitotal EGFr antibody raised in rabbit. The relative contribution of phosphorylated-EGFr and total EGFr bands was calculated using the computer software Scion Image on scanned films. Arbitrarily, the P-EGFr/EGFr ratio in control mice (-EGF) was taken as 1.

frozen sections of skin tissue (data not shown). In terms of concentration per milligram of protein, the amount of ganglioside increased about 30% at the adult stage (1-day-old skin: 0.185 nmol/mg protein; adult skin: 0.239 nmol/mg protein). The ganglioside pattern observed in skin from adult mice was very similar to that previously described by Inoue et al. [2002]. Also, these authors did not observe an accumulation of GM3 ganglioside in skin tissue from Sial-T2/GalNAc-T double knockout mice. In addition, it was previously reported that Sial-T1$^{-/-}$ mice do not express gangliosides of the *a*- and *b*-series [Yamashita et al., 2003].

DISCUSSION

The present study shows an enhanced increase of EGFr phosphorylation, after EGF stimulation, in skin from Sial-T2 knockout and Sial-T2 and GalNAc-T double knockout mice. Interestingly, these results inversely correlate with those previously described [Zurita et al., 2001, 2004]. While the ectopic expression of Sial-T2 synthase in CHO-K1 cells lead to an inhibition of tyrosine phosphorylation of EGFr in response

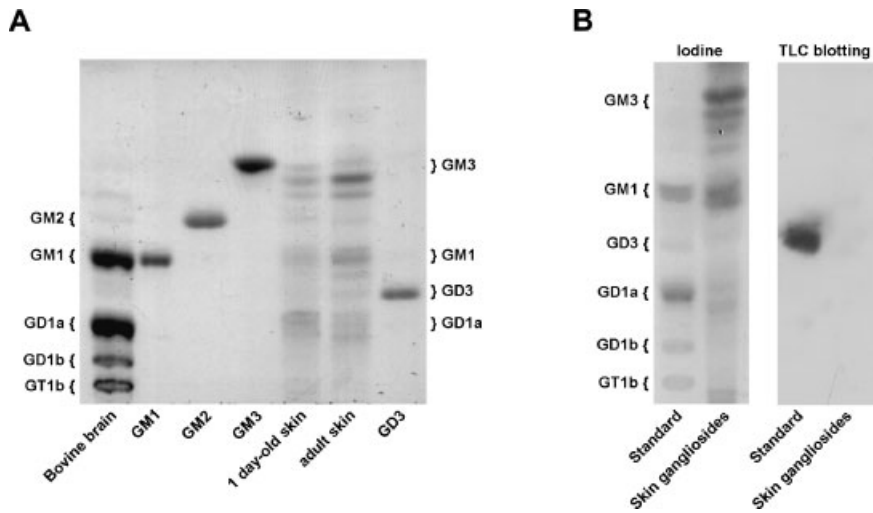

Fig. 7. Analysis of ganglioside expression in skin from normal newborn (1-day-old skin) and adult (adult skin) mice. **A**: Gangliosides derived from 120 mg (wet weight) of skin tissue were applied. Thin layer chromatography was performed with control gangliosides from bovine brain and purified GM1, GM2, GD3, and GM3 gangliosides. A solvent system of chloroform:-methanol:0.2% $CaCl_2$ (60:36:8 v/v) and orcinol spray for detection were used. **B**: Standard gangliosides [GM1, GD3 (0.28 nmol), GD1a, GD1b, and GT1b] and gangliosides derived from 120 mg (wet weight) of adult skin tissue were separated by HPTLC using the solvent system described in A and then blotted to a PVDF membrane, as explained in Materials and Methods. Before blotting, gangliosides from skin tissue were visualized by exposure of the plate to iodine vapours (iodine, left panel). Immunostaining was done using the monoclonal antibody R24 that recognize the ganglioside GD3 and the chemiluminescence detection kit (TLC blotting, right panel).

to EGF, in Sial-T2$^{-/-}$ mice there is a marked tyrosine phosphorylation of EGFr.

Qualitative analysis of gangliosides in skin from normal newborn and adult mice indicate that GM3, GM1, and GD1a are the major gangliosides expressed in skin at both stages. The lacking expression of ganglioside GD3 in skin from adult wild-type mice was undoubtedly confirmed by immunological procedures. This result is in agreement with a recent report demonstrating by RT-PCR that GD3 synthase gene is not expressed in skin from adult mice [Yamada et al., 2005]. The analysis of the ganglioside expression pattern in mice skin strongly suggests that GD3 expression in this tissue might not be a primary factor to modulate EGFr phosphorylation in Sial-T2 knockout and Sial-T2 and GalNAc-T double knockout mice. In addition, the expression of gangliosides from the *a*-series (GM3, GM1, and GD1a) might not also be involved in the regulation of EGFr phosphorylation since the phosphorylation of EGFr after EGF stimulation in Sial-T1$^{-/-}$ mice was comparable to that observed in skin from wild-type mice. We cannot entirely rule out the possibility that Sial-T2 expressed in skin from newborn mice is catalyzing the synthesis of unidentified products (i.e., glycoproteins or glycolipids), which could modulate the activity of the EGFr. In this regard, it has been reported the molecular cloning and expression of a α2, 8-sialyltransferase with activity toward sialylated glycoproteins and α2,3-sialylated glycosphingolipids, such as α2,3-sialylparagloboside and GM3 [Yoshida et al., 1995].

Sial-T2 gene knockout mice exhibit various defects such as thermal hyperalgesia, mechanical allodynia, decreased response to prolonged noxious stimulation [Handa et al., 2005] and reduced regeneration of axotomized hypoglossal nerves [Okada et al., 2002]. In addition, Sial-T2/GalNAc-T double knockout mice exhibit refractory skin injury associated with peripheral nerve degeneration [Inoue et al., 2002]. These and other unknown factors might also be involved in the modulation of EGFr activity. The brain and spinal cords ganglioside pattern of Sial-T2$^{-/-}$ mice is devoid of the *b*-series structures, GD3, GD1b, and GT1b, and is dominated by the *a*-series gangliosides, GM1 and GD1a [Kawai et al., 2001; Handa et al., 2005]. Thus, it should be also taken into consideration that changes in ganglioside expression both in brain and in other tissues may regulate factors, which lastly by systemic distribution could modulate EGFr activity in

skin. Molecular mechanisms involved in the in vivo regulation of EGFr remain to be investigated.

Going beyond the modulation of EGFr phosphorylation by gangliosides in cells from skin, as shown in this work, it would be of interest to explore whether the qualitative change in ganglioside expression is responsible for the different EGF-dependent physiological responses observed throughout development, as previously suggested [Miljan and Bremer, 2002]. Thus, it is known that EGF can cause different physiological responses of neural stem cells at different times of development [Tagami et al., 2002]. Although it was suggested that the change in EGF signal response is due to the EGFr expression level [Burrows et al., 1997], striking changes in ganglioside composition during the development and differentiation of neurons have been described [Maccioni et al., 1999, 2002].

Homozygous loss-of-function mutation of Sial-T1 and targeted disruption of this and other genes encoding glycosyltransferases involved in glycolipid biosynthesis has yielded new insights into the functional and biological roles of glycosphingolipids [Proia, 2003; Simpson et al., 2004]. So far, results have provided compelling evidences for the critical and essential role of gangliosides both in physiological and pathological processes. In this regard, we described enhanced insulin sensitivity in mice lacking ganglioside GM3. A basis for the increased insulin sensitivity in the mutant mice was found to be enhanced insulin receptor phophorylation in skeletal muscle [Yamashita et al., 2003].

In conclusion, the studies shown in this work provide, for the first time, an approach for studying both the molecular mechanisms involved in the in vivo modulation of EGFr function by gangliosides and the physiological relevance of this regulation.

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