Modulation of epidermal growth factor receptor phosphorylation by endogenously expressed gangliosides

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The effect of changing the ganglioside composition of Chinese hamster ovary K1 cells on the function of the epidermal growth factor receptor (EGFr) was examined by studying the signalling pathway generated after the binding of epidermal growth factor (EGF) both in cells depleted of glycosphingolipids by inhibiting glucosylceramide synthase activity and in cell lines expressing different gangliosides as the result of stable transfection of appropriate ganglioside glycosyltransferases. After stimulation with EGF, cells depleted of glycolipids showed EGFr phosphorylation and extracellular signal-regulated protein kinase 2 (ERK2) activity as parental cells expressing GM3 [ganglioside nomenclature follows Svennerholm (1963) J. Neurochem. **10**, 613–623] or as transfected cells expressing mostly GM2 and GD1a as the result of stable transfection of UDP-GalNAc:LacCer/GM3/GD3 *N*-acetylgalactosaminyltransferase. However, cells stably transfected with CMP-NeuAc:GM3 sialyltransferase and expressing GD3 at the cell surface showed both decreased EGFr phosphorylation and ERK2 activation after stimulation with EGF. Results suggest that changes in the ganglioside composition of cell membranes might be important in the regulation of the EGF signal transduction.

Key words: CHO-K1 cells, glycolipid depletion, glycolipid glycosyltransferases, ERK2, insulin receptor, signal transduction.

INTRODUCTION

Gangliosides, glycolipids containing sialic acid, are involved in the regulation of cell proliferation and differentiation. Tumour cells or oncogene-transformed cells show differences in ganglioside composition in comparison with non-transformed cells. Usually, oncogenic cell transformation is associated with a loss of gangliosides containing complex oligosacharides (GM1 and GD1a) (in this paper, gangliosides are named in accordance with [1]) and with an increase in content of simple gangliosides (GM3 and GD3) [2–6].

On the basis that the bulk of the cell's ganglioside is membrane bound, it has been speculated that gangliosides participate in cell-surface events such as receptor function and cell–cell interaction; the effect of adding gangliosides to cells has been examined at different levels. Added GM1 and GQ1b promote neurite outgrowth of neuroblastoma and phaeochromocytoma cells and of primary neuronal cultures [7–9]. Added GM1 increases DNA synthesis in U-1242 MG glioma cells and rat astrocytes in serum-free medium [10,11] and binds to TrkA, potentiating the inductive action of nerve growth factor in PC12 cells [12]. In contrast, added gangliosides inhibit the growth of human glioma cells [13]. Cumulative evidence indicates that gangliosides modify the biological effects of several trophic factors *in vivo* and *in vitro* as well as the mitogenic signalling cascades that these factors generate [14]. It was observed that GM3 and GM1 inhibit the PDGF (platelet-derived growth factor)-stimulated DNA synthesis in the glioma cell line U-1242 MG by inhibiting the receptor dimerization, a critical step necessary for the propagation of stimuli [15,16]. Exogenous gangliosides inhibit the tyrosine protein kinase activity of the epidermal growth factor receptor (EGFr) in the human epidermoid carcinoma cell lines KB and A431 [17] and an increased autophosphorylation of EGFr and epidermal growth factor (EGF)-stimulated cellular proliferation was associated with a decrease in endogenous gangliosides in mutant Chinese hamster ovary (CHO) cells [18]. It has been reported that added GM3 accelerated EGFr dephosphorylation in isolated A431 cell membranes by activating EGFr-directed protein tyrosine phosphatases, suggesting a novel mechanism for the regulation of cellular protein tyrosine phosphatases [19,20].

The precise molecular mechanisms by which added gangliosides influence cell behaviour remain to be clarified. A limitation on this is a lack of knowledge about the type of association that added gangliosides form with the cell membranes. In the present study we investigated the activity of human EGFr in CHO-K1 cell lines depleted of glycolipids by inhibiting glucosylceramide synthase activity with racemic *threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidinopropan-1-ol, HCl (PPPP) or expressing different gangliosides due to stable transfection of appropriate ganglioside glycosyltransferases (a representation of ganglioside synthesis is shown in Scheme 1). We have found that cells with a low glycolipid content showed EGFr phosphorylation levels and extracellular signal-regulated protein kinase 2 (ERK2) activity similar to those of the parental cells expressing mainly GM3 or cells expressing mostly GM2 and GD1a at the cell surface as the result of stable transfection with UDP-GalNAc:LacCer/GM3/GD3 *N*-acetylgalactosaminyltransferase (GalNAc-T). In contrast, cells stably transfected with CMP-NeuAc:GM3 sialyltransferase (Sial-T2) and expressing

Abbreviations used: CHO, Chinese hamster ovary; EGF, epidermal growth factor; EGFr, EGF receptor; ERK, extracellular signal-regulated protein kinase; GalNAc-T, UDP-GalNAc:LacCer/GM3/GD3 *N*-acetylgalactosaminyltransferase; HA, haemagglutinin; HPTLC, high-performance TLC; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; PPPP, racemic *threo*-1-phenyl-2-hexadecanoylamino-3-pyrrolidinopropan-1-ol,HCl; Sial-T2, CMP-NeuAc:GM3 sialyltransferase; gangliosides are named in accordance with [1].

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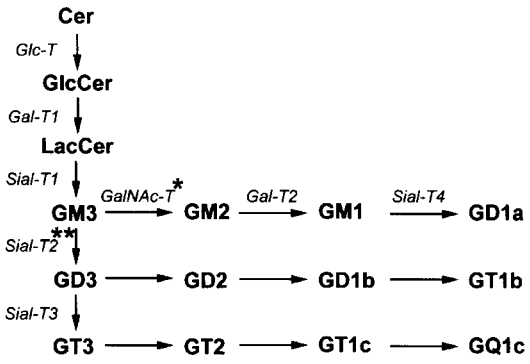

Scheme 1 Biosynthesis of a-, b- and c-series gangliosides

This scheme [40] indicates the pathways opened by transfection of GalNAc-T (*) or Sial-T2 (**) to the parental cells lacking these activities and expressing only GM3. Abbreviations: Cer, ceramide; GlcCer, glucosylceramide; LacCer, lactosylceramide.

mostly GD3 at the cell surface showed both decreased EGFr phosphorylation and ERK2 activation after stimulation with EGF.

MATERIALS AND METHODS

Cell clones and transfection

CHO-K1 cells (A.T.C.C., Manassas, VA, U.S.A.), clone 7 (a stable GalNAc-T transfectant expressing gangliosides GM3, GM2, GM1 and GD1a [21]), clone 18 (a stable Sial-T2 transfectant expressing the ganglioside GD3 [22]) and clone IST2A (expressing the gangliosides GM3 and GD3 by stable transfection of chicken Sial-T2 under the control of an ecdysone-inducible promoter [22]) were grown in DMEM (Dulbecco's modified Eagle's medium) containing 10% (v/v) FCS (fetal calf serum) (Gibco BRL, Gaithersburg, MD, U.S.A.) for 12 h at 37 °C in air/CO_2 (19:1). Each dish of these cell clones was transfected with 1 μg of pcDNAIII-HA-MAPK (HA-ERK2) [23] (in which HA stands for haemagglutinin and MAPK for mitogen-activated protein kinase) or pLCNX-EGFr (human EGFr), a gift from Dr Silvio Gutkind (National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, U.S.A.) or both together with the use of LIPOFECTAMINE (Gibco BRL). After 12–15 h the cells were stimulated with human EGF at the time and concentration indicated in each experiment, washed with cold PBS and harvested for immunoprecipitation, Western blotting or enzyme activity determinations with 10 mM Tris/HCl buffer, pH 7.2, containing 0.25 M sucrose.

Inhibition of glycolipid synthesis with PPPP

Inhibition of glycolipid synthesis with PPPP (Matreya, Pleasant Gap, PA, U.S.A.) was performed as described [21]. Wild-type CHO-K1 cells in culture were treated for different periods with 2 μM PPPP added to the culture medium. Inhibition of glycolipid synthesis was monitored through the analysis of the cellular ganglioside content by high-performance TLC (HPTLC) and orcinol staining [24].

Immunoprecipitation

For immunoprecipitation of EGFr, transfected cells were lysed for 60 min on ice with buffer A [50 mM Tris/HCl (pH 7.2)/0.6% (v/v) Triton X-100/140 mM NaCl/3 μg/ml leupeptin/1 mM PMSF/3 μg/ml aprotinin/1 mM EDTA/0.05% sodium azide]. Lysates were preabsorbed with Protein G–agarose beads [75% (w/v) suspension, washed with buffer A before use] (Pharmacia-Biotech, Uppsala, Sweden) for 60 min at room temperature and were then incubated overnight on a rotating wheel at 4 °C with monoclonal antibody anti-EGFr (diluted 1:50) and with 50 μl of Protein G–agarose beads [25]. Beads were pelleted by centrifugation at 2500 ***g*** for 10 s, washed five times at 4 °C with buffer A, three times with buffer B (100 mM phosphate buffer, pH 7.2, containing 10 mM EDTA) and resuspended in 50 μl of buffer B for SDS/PAGE and immunoblotting.

For ERK2 immunoprecipitation, HA-ERK2-transfected CHO-K1 cells were washed with cold PBS and then lysed at 4 °C in a buffer containing 20 mM Hepes, pH 7.5, 10 mM EGTA, 40 mM β-glycerophosphate, 1% (v/v) Nonidet P40, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 2 mM vanadate, 1 mM PMSF and 20 μg/ml leupeptin. Lysates were clarified by centrifugation at 13000 ***g*** for 5 min at 4 °C; the supernatants were incubated with a 1:300 dilution of the monoclonal antibody 12CA5 (anti-HA) for 4 h at 4 °C. The immunocomplexes were recovered with Protein G–Sepharose by centrifugation. The pellets were washed three times with PBS containing 1% (v/v) Nonidet P40 and 2 mM vanadate, once with 100 mM Tris/HCl, pH 7.5, containing 0.5 M LiCl, and once in kinase reaction buffer [23].

SDS/PAGE and immunoblotting

Cell homogenates and immunoprecipitates were resolved by SDS/PAGE [13% (w/v) gel] under reducing conditions [20% (v/v) 2-mercaptoethanol] [26]. Proteins were transferred electrophoretically to nitrocellulose membranes [27] for 1 h at 300 mA. Protein bands in nitrocellulose membranes were revealed by staining with Ponceau S. For immunoblotting, non-specific binding sites on the nitrocellulose were blocked with 2.5% (w/v) BSA/2.5% (w/v) polyvinylpyrrolidone 40 in Tris-buffered saline [400 mM NaCl/100 mM Tris/HCl (pH 7.5) buffer]. Anti-HA polyclonal antibody (BAbCO, Richmond, CA, U.S.A.), anti-phosphoERK(s) polyclonal antibody (New England Biolabs, Beverly, MA, U.S.A.) and anti-ERK(s) polyclonal antibody K280 (a gift from Dr Silvio Gutkind) were used at a dilution of 1:1000. The anti-phosphotyrosine antibody PY20 was used at a dilution of 1:600. Bands were detected by Protein A coupled to horseradish peroxidase combined with the enhanced chemiluminescence (Renaissance) detection kit (NEN Life Science) and Kodak Biomax MS films. The molecular masses were calculated on the basis of calibrated standards (Gibco BRL) run in every gel. The relative contributions of individual bands were calculated with the computer software Scion Image on scanned films.

Metabolic labelling, lipid extraction and chromatography

Cells at 1 day in culture [$(2–3) \times 10^5$ cells per 35 mm dish] were labelled with 40 μCi/ml [^{3}H]galactose for 12 h. After being washed three times with cold PBS, cells were scraped from the plate and pelleted by brief centrifugation. Lipids were extracted from the cell pellet with chloroform/methanol (2:1, v/v). The lipid extract was freed of water-soluble contaminants by passage through a Sephadex G-25 column. Total radioactivity incorporated into lipids was determined in the eluate. The lipid extract was supplemented with appropriate amounts of standard gangliosides and chromatographed on HPTLC plates (Merck, Darmstadt, Germany) with chloroform/methanol/0.25% $CaCl_2$ (60:36:8, by vol.) as solvent. Standard gangliosides were revealed by exposing the plate to iodine vapour. Routinely, 15000–20000 c.p.m. was spotted on each lane. Radioactive gangliosides were detected by fluorography after dipping the plate into 0.4%

melted 2,5-diphenyloxazole in 2-methylnaphthalene and exposing it to a radiographic film at −70 °C, usually for 4–6 days [28].

ERK2 kinase assay

ERK2 activity was assayed in a reaction buffer [12.5 mM Mops (pH 7.5)/12.5 mM β-glycerophosphate/7.5 mM $MgCl_2$/0.5 mM EGTA/0.5 mM NaF/0.5 mM vanadate] containing 2 μCi of [γ-^{32}P]ATP, 20 μM ATP and 1.5 mg/ml myelin basic protein (MBP) (Sigma-Aldrich). After 30 min at 30 °C, reactions were terminated by the addition of 25 μl of 5 × Laemmli buffer [26]. Samples were heated at 95 °C for 5 min, separated by SDS/PAGE [13% (w/v) gel] and transferred to nitrocellulose. The nitrocellulose membranes were stained with Ponceau S (Sigma-Aldrich) to reveal substrate bands, which were cut and exposed to an X-ray film for autoradiography. Then radioactivity in the band or bands was quantified by scintillation counting. The zone of nitrocellulose membrane containing HA-ERK2 protein was Western blotted with the polyclonal anti-ERK(s) antibody K280 [23]. The activity of ERK2 was determined as a ratio between the radioactivity incorporated into MBP (c.p.m.) and the corresponding staining of HA-ERK2 was revealed by Western blotting and quantified with the computer software Scion Image.

EGF binding assay

Cells grown in a 24-well plate (80000 cells per well) with 200 μl of DMEM were incubated with different concentrations (0.1–5 nM) of ^{125}I-EGF for 2 h at 4 °C in the absence (total binding) or in the presence (non-specific binding) of 250 nM unlabelled EGF, and washed three times with cold PBS. Proteins were solubilized in 300 μl of 1 M NaOH and processed for the determination of ^{125}I radioactivity in a γ-counter. The computer program GraFit 2.11 was used for K_d determination.

RESULTS

Cell line characterization

CHO-K1 cell lines expressing different gangliosides were generated by transfection with either GalNAc-T or Sial-T2. CHO-K1 cells express predominantly the ganglioside GM3, as is shown in the pattern of radioactive lipids metabolically labelled from [^{3}H]galactose for 12 h (Figure 1). Cells stably transfected with the cDNA encoding the chicken Sial-T2 under the control of the human elongation factor promoter (clone 18 [22]) synthesize mostly GD3 and GT3 and accumulate practically no GM3 (Figure 1). To create conditions under which only a fraction of GM3 was used for further sialylation, we stably expressed chicken Sial-T2 under the control of an ecdysone-inducible promoter (clone IST2A [22]). Figure 1 shows that the minimal Sial-T2 activity expressed in the absence of the inductor ponasterone converted approx. 40% of GM3 into GD3. In contrast, CHO-K1 cells stably expressing the human full-length GalNAc-T cDNA (clone 7 [21]) synthesize the a-series gangliosides GM2, GM1 and GD1a because of the constitutive expression in these cells of the enzymes involved in the synthesis of GM1 and GD1a [28] (Figure 1).

To obtain cells with a decreased content of all glycosphingolipid classes, wild-type CHO-K1 cells were treated with PPPP, a potent inhibitor of ceramide glucosyltransferase and hence of the synthesis of glucosylceramide and of more complex glycolipids [29]. Exposure of cells to 2 μM PPPP in the culture medium for 5 days led to a 95% decrease in GM3 content with respect to control cells (Figure 2).

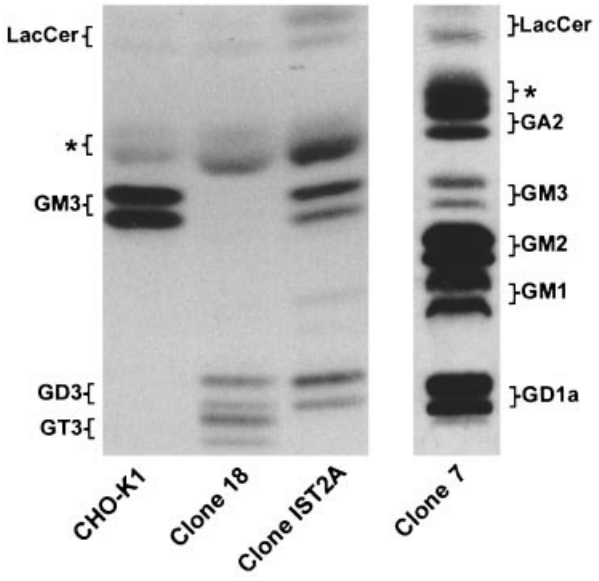

Figure 1 Metabolic labelling of glycolipids of parental cells and of stably transfected CHO-K1 cell clones

Parental cells and clones 18, IST2A and 7 were metabolically labelled with [^{3}H]Gal for 12 h. Lipid extracts were prepared, purified, chromatographed and subjected to HPTLC fluorography as indicated in the Materials and methods section. Asterisks refer to a radioactive non-glycolipid compound previously characterized as phosphatidylcholine [28]. The positions of co-chromatographed glycolipid standards are indicated. Abbreviation: LacCer, lactosylceramide.

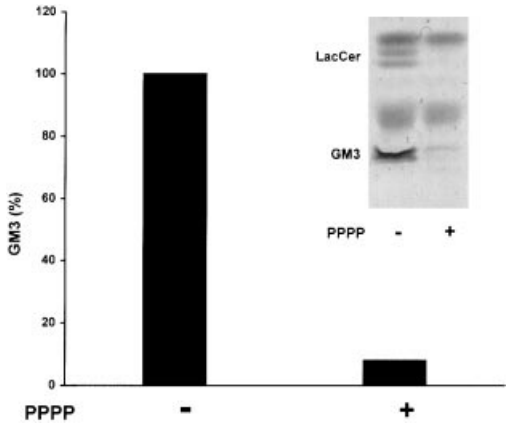

Figure 2 Inhibition of glycolipid synthesis with PPPP

Cells were cultured in complete medium without (−) or with (+) 2 μM PPPP for 5 days. Lipids were extracted from equal mounts of cell protein, purified, separated by HPTLC and stained with orcinol (see inset). The positions of co-chromatographed GM3 and lactosylceramide (LacCer) standards are indicated. The percentage of GM3 under each condition was determined by densitometry of the TLC. The amount of GM3 in control cells (CHO-K1, −PPPP) was taken as 100%.

Expression and functional characterization of the human EGFr

A cDNA encoding the human EGFr was transiently transfected to the cell clones stably expressing the different glycosyltransferases. EGFr expression was confirmed by Western blotting with an antibody directed to its intracellular domain. Although EGFr expression in CHO-K1 cells was below the limit of

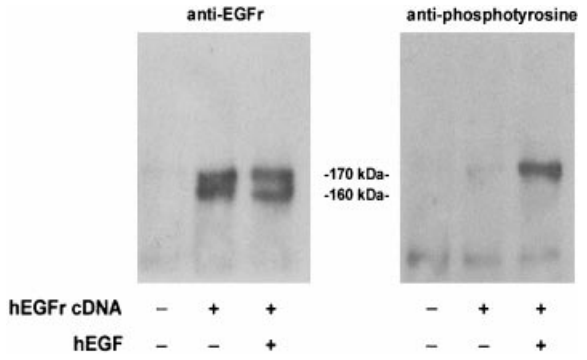

Figure 3 EGF-induced tyrosine phosphorylation of EGFr

Mock-transfected cells (−) or cells transfected with the hEGFr cDNA (+) were incubated in the absence or presence of 30 nM human EGF (hEGF), as indicated, for 5 min. Post-nuclear lysates (70 μg of protein) were subjected to SDS/PAGE [8% (w/v) gel], immunoblotted with anti-EGFr antibody (left panel) or with anti-phosphotyrosine antibody (right panel), and visualized by enhanced chemiluminescence. The positions of glycosylated (170 kDa) and unglycosylated (160 kDa) human EGFr are indicated.

detection [30] (Figure 3, left panel, first lane), the antibody detected the EGFr in transfected cells as two bands, one of approx. 170 kDa and the other of approx. 160 kDa. The digestion of EGFr, before SDS/PAGE, with N-glycanase, which cleaves the asparagine–oligosaccharide bond of N-linked oligosaccharides, decreased the 170 kDa band and increased the 160 kDa band (results not shown), indicating that the former band corresponded to the fully glycosylated form and the 160 kDa band to the unglycosylated form [31].

To determine whether the expressed EGFr was functional, the ability of the EGF to induce receptor tyrosine phosphorylation was examined. Transfected cells were stimulated for 5 min with 30 nM human EGF. No variations in EGFr immunostaining were observed in EGF-stimulated cells (Figure 3, left panel). However, immunoblotting with the anti-phosphotyrosine antibody PY-20 (Figure 3, right panel) clearly demonstrated a stimulation of tyrosine phosphorylation of the receptor by EGF.

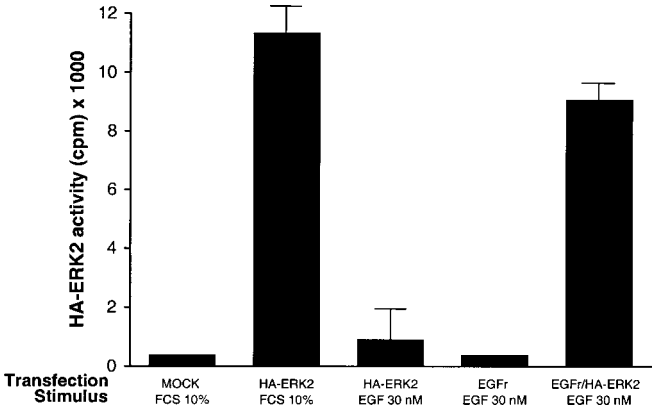

Figure 4 Activation of ERK2 by stimulation with EGF

Cells expressing EGFr or HA-ERK2 or both were stimulated with either 30 nM EGF or 10% (v/v) fetal-calf serum; ERK2 activity was determined 5 min later. In parallel experiments, cells were co-transfected with empty pLCNX (mock-transfected). ERK2 activity is shown as ^{32}P radioactivity incorporated into the substrate MBP. Results are means ± S.D. for three independent experiments.

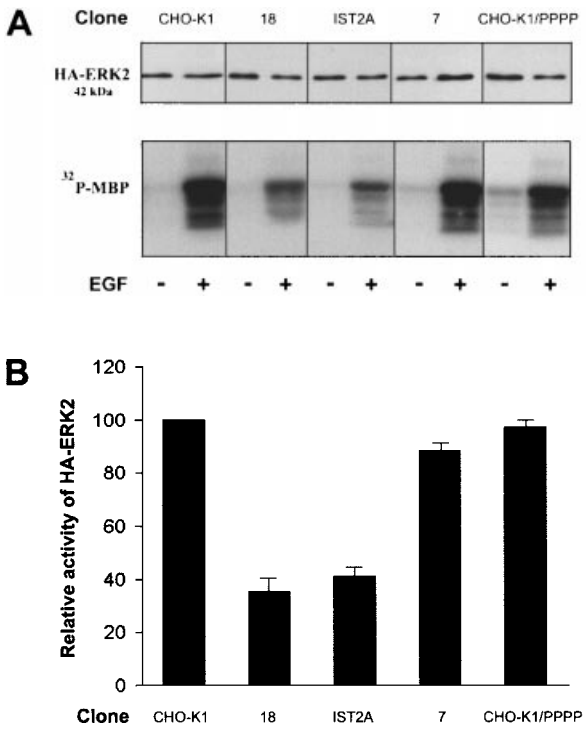

Figure 5 EGFr function in CHO-K1 clones expressing different gangliosides

Parental cells, parental cells treated with PPPP for 5 days, and the indicated cell clones were co-transfected with pCLNX-EGFr and pCDNA-HA-MAPK. Cells were grown for 12 h in serum-free medium and then stimulated with 1 nM EGF for 5 min. Cells were lysed, ERK2 was immunoprecipitated and its activity was determined as indicated in the Materials and methods section. (**A**) Results of a typical experiment showing the expression level of HA-ERK2 as determined by Western blotting (upper panel) and the kinase activity determined by autoradiography (lower panel), in the presence (+) and in the absence (−) of EGF. (**B**) Averaged data (means ± S.D) from three independent experiments. ERK2 activity for wild-type CHO-K1 cells was taken as 100%. Values were normalized to the amount of ERK2 and EGFr protein expressed in each clone, as estimated by Western blotting and densitometric scanning.

Phosphorylation of EGFr was negligible in the absence of added EGF.

To examine whether the binding of EGF to the expressed EGFr generated a stimulus able to couple with and activate ERK2, an intermediate in the EGF signal cascade, cells were co-transfected with EGFr and an influenza virus HA-epitope-tagged form of ERK2. Control cells transfected with HA-ERK2 responded to 10% (v/v) fetal-calf serum with an approx. 50-fold increase in ERK2 activity (Figure 4). Neither HA-ERK2-transfected nor EGFr-transfected cells responded to 30 nM EGF. However, cells co-expressing both EGFr and ERK2 responded to 30 nM EGF with an approx. 40-fold stimulation of ERK2 activity, indicating that the expressed EGFr and HA-ERK2 were functionally coupled (Figure 4). Mock-transfected cells, lacking EGFr, showed no stimulation.

EGFr function in cell lines expressing different gangliosides

Having shown that ERK2 activity is useful in sensing the signal transduction activity of the EGFr, we next examined the behaviour of the EGFr in the different glycolipid environment provided by the membranes of the cell lines under study by measuring ERK2 activity.

The ERK2 response at 5 min after addition of 1 nM EGF is shown in Figure 5. The activity of ERK2 was essentially

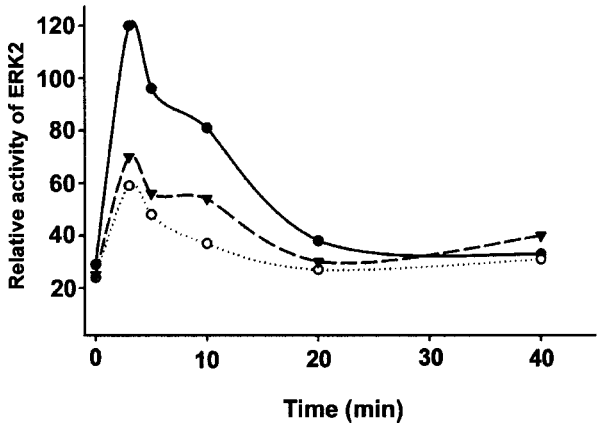

Figure 6 Time course of activation of ERK2 by EGF

Parental CHO-K1 cells (●), clone 18 cells (▼) and clone IST2A cells (○) were transiently transfected with pCLNX-EGFr and pCDNA-HA-MAPK and then maintained in a serum-free medium for 12 h. EGF (1 nM) was added to the medium, and the cells were harvested at 0, 3, 5, 10, 20 and 40 min for ERK2 activity determination as described in the Materials and methods section.

comparable in clones expressing GM3 (CHO-K1 cells) and in GM3, GM2, GM1 and GD1a (clone 7). Similar results were also obtained in CHO-K1 cells with a generalized decrease in glycolipid expression, as shown in Figure 2 (CHO-K1/PPPP). In contrast, in those clones overexpressing the ganglioside GD3 (clones 18 and IST2A) the response of ERK2 activity to EGF was decreased to approx. 45–50% of that of the other GD3-deficient clones. ERK2 activity at 0 nM EGF was very low and was similar in all clones analysed (results not shown).

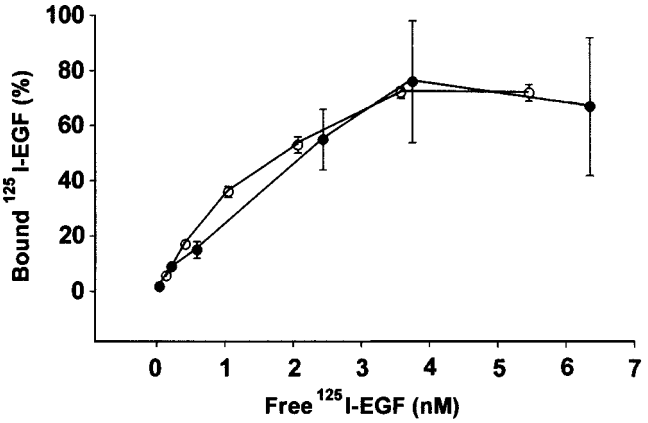

Figure 7 Specific EGF binding in clone 18 cells (●) and parental CHO-K1 cells (○)

Cells expressing EGFr were grown in a 24-well plate with 200 μl of DMEM at 80000 cells per well. Cells were incubated with ^{125}I-EGF at different concentrations in the range 0.1–5 nM for 2 h at 4 °C in the absence (total binding) or in the presence (non-specific binding) of 250 nM unlabelled EGF. Cells were washed, proteins were solubilized and ^{125}I radioactivity was determined in a γ-counter. Specific binding was calculated from the difference between total and non-specific binding. The computer program GraFit 2.11 was used for K_d determination. For clone 18 cells, K_d was 2.1 ± 0.9 nM; for CHO-K1 cells, K_d was 1.8 ± 0.4 nM. Results are means ± S.D. for two experiments.

Time course of activation of ERK2

To examine the possibility of differences in the time course of ERK2 activation in clones overexpressing GD3, ERK2 activity was assayed at different times after adding EGF in the parental cells and in clones 18 and IST2A. Figure 6 shows that the kinetics of ERK2 activation was similar in all these cells, with a maximum at approx. 3–5 min. However, as shown in Figure 5, this activation was lower in GD3-overexpressing cells for the first 20 min after the addition of 1 nM EGF. These results preclude the possibility that the decreased ERK2 activation by stimulation with EGF of GD3-overexpressing clones was due to changes in the kinetics of ERK2 response.

EGF binding analysis

The decreased ERK2 activation in GD3-overexpressing clones raises the possibility of a decreased affinity of EGFr for EGF in these cells. To examine this possibility, the binding of ^{125}I-labelled EGF to EGFr in clone 18 and in parental CHO-K1 cells was examined. As shown in the plot of bound EGF against free EGF in Figure 7, the binding of ^{125}I-labelled EGF to clone 18 and parental cells was essentially identical, as were the determined K_d values. This indicates that the decreased ERK2 activation in GD3-expressing cells was not due to changes in the affinity of EGF for EGFr.

Analysis of early signalling events mediated by EGFr in GD3-expressing cells

The binding of EGF to its receptor results in the receptor's becoming phosphorylated on tyrosine residues. This allows cytoplasmic proteins containing an SH2 (Src homology 2) domain, such as Src, phosphoinositide 3-kinase, Shc and Grb2, to bind to the phosphotyrosine sequence on the receptor. The recruitment and phosphorylation of Shc (p46/52) and Grb2 represent receptor-proximal steps towards the activation of the Ras–MAPK(s) pathway [32]. Thus an inhibition of the EGFr associated tyrosine kinase activity strongly affects downstream signalling events, such as MAPK(s) activation. To determine whether the EGFr tyrosine kinase activity was modified in GD3-overexpressing cells, wild-type, clone 18 and clone IST2A cells expressing the EGFr were stimulated for 5, 10 and 20 min with 1 nM EGF and the receptor was immunoprecipitated with anti-EGFr antiserum. Proteins were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with anti-phosphotyrosine antibody. The results in Figure 8 show receptor tyrosine phosphorylation both in wild-type and in the two GD3-overexpressing clones (upper panel). However, it was clear that a significant inhibition of EGFr phosphorylation occurred in clone 18 and clone IST2A cells when the bands were normalized to the intensity of the total EGFr band (Figure 8, lower panel). Basal phosphorylation of EGFr was negligible in the absence of stimulation with EGF (Figure 8, upper panel). Figure 8 shows that elevated levels of GD3 in the cell membranes can negatively affect the EGFr-associated tyrosine kinase activity. Taking this result together with that of Figure 5, the simplest interpretation is that the decreased ERK2 activity in GD3-overexpressing clones was a consequence of the decreased tyrosine kinase activity of the EGFr in these cells.

Overexpression of GD3 does not affect insulin receptor function

To investigate whether the effect of overexpressed GD3 on EGFr behaviour was restricted to EGFr or whether it included other cell-surface receptors of the tyrosine-specific protein kinase

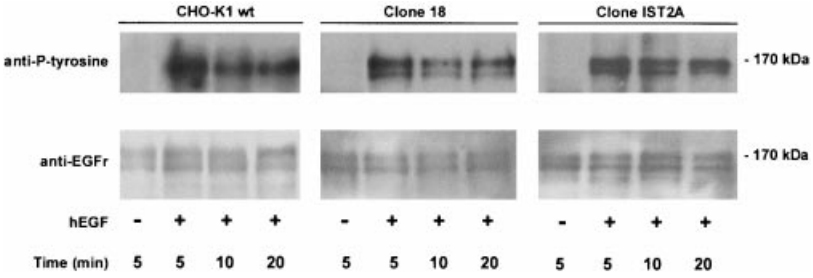

Figure 8 Analysis of EGFr phosphorylation in parental CHO-K1 cells, clone 18 cells and clone IST2A cells

Cells transiently expressing human EGFr were maintained in serum-free medium for 12 h. EGF (1 nM) was added to the medium and cells were harvested at 5, 10 and 20 min. EGFr was immunoprecipitated; proteins were resolved by SDS/PAGE, transferred to a nitrocellulose membrane and immunoblotted with the anti-phosphotyrosine antibody PY-20 (upper panel). Antibodies were then removed by treatment of the membrane with 1 M NaOH for 5 min and the total EGFr was determined by immunoblotting with anti-EGFr (lower panel). In both cases antibody binding was detected by the enhanced chemiluminescence technique. The position of EGFr (approx. 170 kDa) is indicated. Abbreviation: hEGF, human EGF.

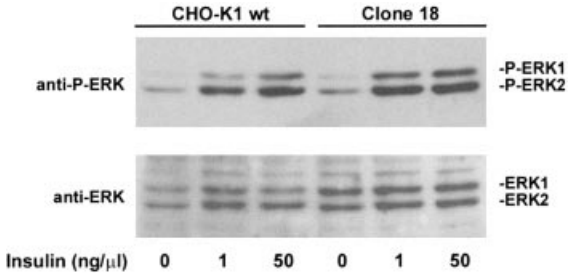

Figure 9 ERK1/2 activation by insulin in clone 18 cells and wild-type (wt) CHO-K1 cells

Cells maintained in serum-free medium for 12 h were stimulated with 1 and 50 ng/μl insulin. After 5 min, cells were harvested; proteins were resolved by SDS/PAGE [10% (w/v) gel] and transferred to a nitrocellulose membrane. Phospho-ERK(s) (active form) was identified with the specific polyclonal antibody anti-(phospho-ERK) and subsequent chemiluminescence (upper panel). First and second antibodies were then removed by treatment of the membrane with 1 M NaOH for 5 min and total ERK(s) were revealed with the polyclonal antibody K-280 (anti-ERK, lower panel). The positions of ERK1 (44 kDa) and ERK2 (42 kDa) are indicated.

family, we explored the function of the insulin receptor, which is expressed endogenously in CHO-K1 cells. As in the EGFr studies, we measured ERK1/2 activation to sense the signal transduction activity of the insulin receptor after the stimulation of cells with insulin. The endogenous activity of ERK(s) was examined at two insulin concentrations (1 and 50 ng/μl) by Western blotting with a polyclonal antibody recognizing phospho-ERK(s) (active form). Phosphorylation of ERK1 (44 kDa) and ERK2 (42 kDa) was stimulated 5 min after treatment with insulin both in parental cells and in clone 18 cells (Figure 9). Quantification of phospho-ERK(s) bands and normalization to total ERK(s) protein determined in the same membrane (Figure 9, lower panel) showed clearly that activation of the insulin receptor at the two insulin concentrations was essentially the same in both cell lines. Basal ERK(s) activity (0 ng/μl) was similar in both of the clones analysed.

DISCUSSION

EGFr (ErbB1) is a large transmembrane glycoprotein with ligand-inducible tyrosine kinase activity. The binding of EGF to its receptor results in the receptor's becoming phosphorylated on tyrosine, thus initiating a kinase cascade that results in the activation of 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 ERK(s) kinases [33].

The influence of the lipid environment on the EGFr response to its specific ligand has been examined by adding exogenous gangliosides to cells in culture [17,18]. Although this approach has been widely used to study the effects of gangliosides on cellular systems, a major limitation is that it does not provide knowledge of the nature of the association and therefore the real concentration of the glycolipids properly inserted into the plasma membrane [34]. Our approach was to change the glycolipid composition of the plasma membrane just by changing the ganglioside biosynthetic activity of the cell, while keeping the normal process of membrane insertion and intracellular transport unmodified. In addition, the function of EGFr was examined in cells with a 95% decrease in endogenous GM3 by inhibiting glycolipid synthesis with PPPP, a potent inhibitor of ceramide glucosyltransferase [29].

The activity of ERK2, an intermediate in the EGF signal transduction cascade, was determined to sense the function of the EGFr in the different conditions. When assayed 5 min after EGFr stimulation, a decreased ERK2 activation was detected in those clones overexpressing the ganglioside GD3 (clones 18 and IST2A). The decreased ERK2 activity was not due to an alteration in the affinity of the EGFr to EGF, because the K_d values were essentially the same as in the parental cells. The kinetics of ERK2 activation after treatment of the cells with EGF was very similar in clones overexpressing GD3 and in parental cells, with a maximum at approx. 3–5 min after stimulation. The decreased ERK2 activation in GD3-overexpressing clones was not attributable to a decrease in the content of GM3 by conversion into GD3, because a decrease in GM3 content of up to 95% in PPPP-treated cells did not significantly affect ERK2 activation by EGF. This latter observation is at variance with results reporting an inverse correlation between the level of GM3 expression and EGFr-mediated signal transduction in a mutant CHO cell line (*ldl* D cells) [18] lacking the UDP-Glc 4′-epimerase. Because these cells cannot synthesize UDP-galactose and UDP-GalNAc and therefore galactose-containing glycoconjugates, it is possible that other galactose-containing molecule(s) could modulate the function of EGFr in addition to GM3 ganglioside.

The possibility was considered that changes in glycolipid composition might have affected the intracellular trafficking of EGFr, as reported for other proteins [35]. However, we checked

that the fractions of EGFr at the plasma membrane determined from the binding of ^{125}I-EGF with regard to the total EGFr in the cell were essentially the same in the parental cells and in the different cell clones examined (results not shown).

Results obtained here indicate that in cells overexpressing GD3 ganglioside there was a decreased phosphorylation of tyrosine residues of EGFr in response to EGF. The reduced MAPK activation in these cells after stimulation with EGF (Figure 5) could therefore be attributed to an inhibition of the phosphorylation-dependent binding of SH2-domain signalling proteins, leading to the activation of the Ras–MAPK pathway [32].

We cannot define where in the membrane GD3 exerts its modulatory effect on EGFr. It has been reported that GD3 and EGFr coexist in caveolae [36–38]; however, in a series of experiments not shown here we were unable to co-immunoprecipitate the EGFr with the anti-GD3 monoclonal antibody R24 or to co-immunoprecipitate GD3 with the anti-EGFr antibody in lysates from clone 18. In addition, we have found that the immunocytochemical expression of GD3 was in the form of a punctuate pattern of staining clearly different from the more uniform distribution of the EGFr. These results make it less probable that there is a direct effect of GD3 on EGFr and suggest an indirect effect, perhaps through its interaction with other membrane-associated proteins, as described for the effect of GD3 on mitochondria during apoptosis through a direct interaction with components of the permeability transition pore complex rather than from a perturbation of the properties of cellular membranes [39]. In the system that we have studied, an interesting possibility is that the decreased phosphorylation of the EGFr in GD3-overexpressing cells could have resulted from an increased EGFr-directed tyrosine phosphatase, as with the GM3 activatory effect in human lung adenocarcinoma cells [19,20].

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