Inhibition of EGF-mediated receptor activity and cell proliferation by HK1-ceramide, a stable analog of the ganglioside GM3-lactone

Frauke Alves¹,², Ulrich Borchers³, Holger Keim¹, Rocco Fortte³, Jens Olschimke³, Wolfgang F. Vogel⁴, Hartmut Halfter³, and Lutz F. Tietze³

¹Department of Hematology and Oncology, Georg-August-University, Robert-Koch-Str. 40, 37075 Göttingen, Germany; ²Department of Organic Chemistry, Georg-August-University, Tammannstr. 2, 37077 Göttingen, Germany; ³Department of Laboratory Medicine and Pathobiology, University of Toronto, 1 King’s College Circle, Toronto, ON M5S 1A8, Canada; and ⁴Clinic of Neurology, Westf. Wilhelms-University Münster, Albert-Schweitzer-Str. 33, 48129 Münster, Germany

Received on December 3, 2001; revised on April 3, 2002; accepted on April 3, 2002

Gangliosides have been described as modulators of growth factor receptor activity and subsequent cellular function. Due to the lower-pH environment found in tumor cells, gangliosides are thought to be formed (at least to some extent) into their lactone forms. The aim of the study was to analyze the mode of action of the lactone of the ganglioside GM3 on epidermal growth factor (EGF) signaling in human ovarian epidermoid carcinoma A431 cells and cell growth in human oral epidermoid carcinoma KB cells by applying the GM3 lactone analog HK1-ceramide 2, which is stable under hydrolytic conditions. Specific inhibition of EGF-dependent receptor tyrosine phosphorylation was observed by HK1-ceramide 2 at 25 µM, whereas GM3 showed a comparable inhibition at eightfold higher concentrations. In cells exposed to low pH, where GM3 is thought to form its lactone to a higher extent, addition of GM3 showed no further inhibitory effect on EGF-dependent receptor phosphorylation. Similarly to GM3, HK1-ceramide 2 does not affect binding of ¹²⁵I-EGF to the cell surface receptor. EGF-dependent growth of KB cells was also found to be inhibited by HK1-ceramide 2 at much lower concentrations compared to GM3. In conclusion, our results indicate that the GM3 lactone analog HK1-ceramide 2 is a specific inhibitor of EGF receptor function and is more potent in reducing EGF-dependent tyrosine phosphorylation of the receptor in A431 cells and in inhibiting EGF-dependent growth of KB cells compared to GM3.

**Key words:** EGF receptor-signaling /ganglioside GM3/ ganglioside lactone/proliferation/tyrosine kinase activity

**Introduction**

The involvement of gangliosides in tumor development and progression, for example, cell proliferation, migration, and adhesion, has been widely described (Oettgen, 1989; Lee and Lee, 1995). In addition to proteins and lipids, carbohydrates—of which gangliosides constitute a large part—are essential elements of the cell surface. Gangliosides are neuraminic acid–containing glycosphingolipids that are responsible for a variety of biological recognition processes. They are anchored in the lipid bilayer of the membrane by the ceramide portion with the carbohydrate moiety being exposed on the outside of the cell. The ganglioside pattern on the surface of tumor cells is different from nontransformed cells. Furthermore, the amount of GM3, GM2, and GD3 has been found, for instance, to be increased on malignant melanomas when compared to melanocytes (Zhang et al., 1997; Livingston et al., 1997). GM3 is also highly expressed in melanoma cells with metastatic potential (Tardif et al., 1996). It has been suggested that an equilibrium exists between the gangliosides and their lactone forms, for example, GM3 and its lactone 1, by reaction of the sialic acid moiety with a hydroxyl group of an adjacent sugar in the molecule (Figure 1). These ganglioside-lactones are thought to be formed on malignant cells probably due to their lower pH environment and may therefore play an important role as tumor-associated antigens (Kawashima et al., 1994). They are unstable under physiological conditions due to hydrolysis.

It has been demonstrated that the GM3-lactone 1 is more immunogenic in comparison to GM3. The antimelanoma GM3 antibody M2590 exhibits a high affinity for GM3-lactone 1 but a low affinity for GM3 (Nores et al., 1987). In particular, the ganglioside-lactone GM3 has become of special interest because it has been identified in mammary and gastric tumors (Tekiwa and Diatlovitskaia, 1993) and was found to bind to influenza virus hemagglutinin (Sato et al., 1999). Furthermore, gangliosides extracted from mullet milt were identified as GM3, GM3-lactone 1, GM3 methylester, and 9-O-acetyl GM3 (Zhu et al., 1999).

Ganglioside GM3 and, to a lesser extent, GM1 but not other gangliosides have been described to modulate cell growth through inhibition of epidermal growth factor (EGF) receptor–associated tyrosine kinase activity (Bremer et al., 1986). On binding of EGF to its receptor, the receptor becomes phosphorylated on tyrosines, allowing cytoplasmatic proteins...
to bind to the phosphotyrosine sequence on the receptor leading to the phosphorylation of mitogen-activated protein kinases (MAP kinases) (Cobb and Goldsmith, 1995).

The effect of GM3 appears to be mediated directly by inhibition of EGF receptor autophosphorylation and dimerization (Bremer et al., 1986; Bremer, 1994) rather than by acting on the intracellular intermediates of EGF receptor signaling (Rebbaa et al., 1996). Therefore, ganglioside analogs that inhibit receptor associated tyrosine kinases have been applied for the inhibition of tumor growth and metastasis (Hakomori, 1996; Suarez-Pestana et al., 1997) and have been shown to modulate the angiogenic response of tumors (Alessandri et al., 1997). Reduction of GM3 expression in A431 cells by transfection of the sialidase gene or application of a ceramide analog resulted in enhanced EGF receptor activity and induction of growth (Meuillet et al., 1999, 2000).

Recently, we succeeded in the synthesis of the GM3-lactone analog HK1-ceramide 2 (Figure 1) which contains an ether moiety instead of the lactone functionality and is stable under physiological conditions (Tietze and Keim, 1997; Tietze et al., 2000). To our knowledge, HK1-ceramide 2 is the only stable ganglioside-lactone analog synthesized so far and is thus a perfect tool to analyze the mode of action of GM3-lactone 1 formed in vivo.

The goal of this study was to determine if the lactone form of GM3 has a similar inhibitory effect as GM3 itself on EGF-induced cell growth in human epidermoid carcinoma KB cells and EGF receptor tyrosine phosphorylation in human epidermoid carcinoma A431 cells. To accomplish this, the effects of the stable ganglioside-lactone analog HK1-ceramide 2 toward EGF receptor signaling and EGF-mediated cell growth were examined and directly compared to the observed effects of GM3.

Results

Effect of HK1-ceramide 2 on EGF-mediated tyrosine phosphorylation of EGF receptor in cultured intact A431 cells

Because human epidermoid carcinoma A431 cells contain high numbers of EGF receptors (2 × 10⁶) on their surface (Kawamoto et al., 1983), these cells were used in several studies to analyze EGF signaling. The effect of HK1-ceramide 2 as a stable analog of GM3-lactone on EGF receptor phosphorylation was investigated by incubating A431 cells with different concentrations of HK1-ceramide 2 for 3 h and stimulation with 50 ng/ml EGF for 30 min. The intrinsic tyrosine kinase is activated on binding of EGF as demonstrated by western blot analysis immunoblotted with an antiphosphotyrosine antibody (αPY). The phosphorylated band observed at 170 kDa represents the polypeptide chain of the EGF receptor (Figure 2B). A baseline phosphorylation of the EGF receptor was demonstrated (Figure 2A, lane 7) and in the presence of dimethyl sulfoxide (DMSO) alone, a slight increase of EGF receptor autophosphorylation was observed (Figure 2A, lane 6).

EGF-mediated tyrosine phosphorylation of the receptor was inhibited by HK1-ceramide 2 at a concentration of 25 μM (Figure 2A, lane 2, Figure 3, lane 2). This concentration being most effective was found in six independent experiments. Furthermore, a reduced tyrosine phosphorylation in response to EGF after addition of 25 μM HK1-ceramide 2 was detected in proteins with lower molecular weight, in particular of a 116-kDa protein, suggesting that downstream targets of the EGF receptor were also affected by HK1-ceramide 2 (Figure 2A, lane 2; Figure 3, lane 2). The inhibitory effect of 25 μM HK1-ceramide 2 on EGF receptor autophosphorylation was only observed after stimulation with EGF and not in unstimulated cells (Figure 2A, lane 2 in comparison to lane 4). Reblotting of the nitrocellulose membranes with a specific EGF receptor antibody showed equal amounts of receptor being expressed in each assay (Figure 2B). Surprisingly, the inhibitory effect of HK1-ceramide 2 was only observed in a narrow window. Concentrations of HK1-ceramide between 20 – 30 μM showed reduced tyrosine
phosphorylation of the EGF receptor in response to EGF with maximal inhibition at 25 μM concentration (Figure 3). In contrast, concentrations of HK1-ceramide 2 below 20 μM or in the range of 30–100 μM showed no inhibitory effect on tyrosine phosphorylation of the EGF receptor and proteins of lower molecular weight (data not shown, Figure 2, Figure 3). GM3 in a concentration of 25 μM and 50 μM showed no effect on EGF-mediated tyrosine phosphorylation of the receptor (Figure 4).

Effects of GM3 on EGF-mediated tyrosine phosphorylation of EGF receptor in cultured intact A431 cells in dependence of the pH

Moderate inhibition of EGF-mediated tyrosine phosphorylation of the EGF receptor was observed in the presence of 200 μM GM3 at physiological pH values (Figure 5, lane 1, panel B and C), whereas no inhibition was observed in the presence of 25 μM and 50 μM (Figure 4). We next evaluated whether the inhibitory effect of high concentrations of GM3 on EGF receptor phosphorylation might be due to a lactonization of GM3. Therefore, we investigated EGF-dependent receptor tyrosine phosphorylation in A431 cells in the presence of 200 μM GM3 at lower pH values where GM3 lactones are thought to be formed. Surprisingly, decreasing pH values up to pH 5.8 resulted in enhanced constitutive, EGF-independent tyrosine phosphorylation of the receptor (Figure 5A). In contrast, on EGF stimulation the tyrosine phosphorylation of the EGF receptor was reduced in cells exposed to low-pH media in comparison to controls (Figure 5B, lane 1 compared to lane 5). GM3 showed an inhibition of EGF-mediated tyrosine phosphorylation of the EGF receptor (Figure 5, lane 1, panels B and C). Compared to cells exposed to neutral pH and GM3, no further inhibitory effect on EGF-dependent receptor phosphorylation was observed by 200 μM GM3 in cells treated at low pH (Figure 5C, lane 1 compared to lane 5).

Effect of HK1-ceramide 2 on EGF-dependent mitogenesis in KB cells

Because high concentrations of EGF inhibit the growth of A431 cells (Kawamoto et al., 1983), oral epidermoid carcinoma KB cells characterized by the presence of a moderate concentration of a high-affinity EGF receptor are frequently used to analyze EGF-dependent mitogenesis (King and Cuatrecasas, 1982). Therefore, the effect of HK1-ceramide 2 on EGF-dependent growth were studied in KB cells. In the presence of different concentrations of HK1-ceramide 2, 6 μM, 17 μM, and 25 μM KB cell growth was inhibited in a dose-dependent manner to 61%, 82%, and 86%, respectively (Table 1).

Effect of HK1-ceramide 2 on specific binding activity of $^{125}$I-EGF to cell surface receptor

To determine if the change in EGF-mediated tyrosine phosphorylation of the EGF receptor observed after HK1-ceramide 2 incubation was due to an alteration of EGF receptor ligand binding characteristics, EGF competition binding experiments were performed on KB cells treated with HK1-ceramide 2 and on control cells. The binding activities of $^{125}$I-EGF on the cell surface of KB cells grown in normal culture media and in media to which different amounts of HK1-ceramide 2 (6, 11, 25, and 56 μM) were added are shown in Figure 6. No differences were observed in the binding activities obtained from HK1-ceramide 2–treated cells compared to controls.

Discussion

Gangliosides have been described as modulators of cell growth. Changes in the structure, synthesis and cell surface exposure of gangliosides have been described to be associated with tumor progression (Tardif et al., 1996). For example, the lactone form of gangliosides are thought to be formed, at least to some extent, on malignant cells probably due to a lower pH environment of these cells (Kawashima et al., 1994). In particular, the ganglioside GM3 has been found to affect cell growth presumably by inhibition of EGF receptor phosphorylation (Bremer et al., 1986; Rebbaa et al., 1996).

The aim of the study was to determine whether the GM3 lactone 1 has an inhibitory effect on EGF-dependent tyrosine phosphorylation of the receptor and therefore on modulation of cell growth. Because the unmodified GM3 lactone 1 cannot be used due to its instability under physiological conditions, we therefore examined the effect of the GM3 lactone analog HK1-ceramide 2 on EGF receptor signaling in human ovarian
Table 1. Effect of HK1-ceramide 2 on EGF-dependent mitogenesis in KB cells

<table>
<thead>
<tr>
<th>HK1-ceramide (µM)</th>
<th>EGF (1 ng/ml)</th>
<th>OD (450 nm)</th>
<th>Inhibition of mitogenesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>—</td>
<td>—</td>
<td>0.140</td>
<td>—</td>
</tr>
<tr>
<td>—</td>
<td>+</td>
<td>0.473</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>+</td>
<td>0.265</td>
<td>62 (± 8)</td>
</tr>
<tr>
<td>17</td>
<td>+</td>
<td>0.204</td>
<td>81 (± 4)</td>
</tr>
<tr>
<td>25</td>
<td>+</td>
<td>0.185</td>
<td>86 (± 1)</td>
</tr>
</tbody>
</table>

Presented values are the arithmetic mean of five determinations.

Fig. 6. Effects of the presence or absence of different quantities of HK1-ceramide 2 on the specific binding capacity and affinity of 125I-EGF to the EGF receptor in A431 cells. The cell monolayers were washed, and 125I-EGF with a specific activity of 2.8 × 10^7 cpm/µg was added to each well and incubated at 4°C for 2 h. Nonspecific binding of EGF was determined by preincubation of a 500–1000-fold excess of cold EGF in four separate wells with different quantities of 125I-EGF (0.25, 0.5, 1, 3.5, 10, 15, and 20 ng/ml 125I-EGF). Cells were washed and incubated with 1 N NaOH at 25°C for 1 h, and the radioactivity associated with the cell monolayers was counted with a gamma counter. The effect of HK1-ceramide 1 on the specific binding of 125I-EGF is presented by the method of Scatchard (1949). TB, total binding; SB, specific binding; NSB, nonspecific binding; squares, 125I-EGF; no glycolipid; circles, 125I-EGF, 25 µM HK1-ceramide 2; triangles, 125I-EGF, 6 µM HK1-ceramide 2; upside-down triangles, 125I-EGF, 11 µM HK1-ceramide 2; diamonds, 125I-EGF, 56 µM HK1-ceramide 2. Total volume of reaction mixture was 100 µl.

Epidermoid carcinoma A431 cells and mitogenesis in human oral epidermoid carcinoma KB cells. Compared to GM3 lactone, HK1-ceramide 2 contains an ether moiety instead of the lactone functionality and is stable under physiological conditions (Tietze and Keim, 1997). As controls, we also used GM3 itself and the GM3 lactone ether analog (HK2-ceramide 3) with the β configuration at the 2-position of the neuraminic acid moiety. This compound shows a different structural arrangement as compared to GM3 lactone 1.

A431 cells were used for the analysis of EGF receptor signaling because these cells have an elevated endogenous concentration of EGF receptors and have therefore been used in a variety of receptor phosphorylation studies concerning the effect of GM3 (Bremer et al., 1986). On stimulation of EGF, the receptor becomes phosphorylated on tyrosine residues that bind to specific SH2 domain–containing molecules, which are often themselves phosphorylated by the receptor, thus initiating the signaling cascade. Here, we demonstrate that HK1-ceramide 2 at a concentration of 25 µM decreases ligand-dependent activation of EGF receptor in A431 cells without affecting EGF receptor content. This effect was only observed in a narrow range of concentration. In addition, tyrosine phosphorylation of proteins with lower molecular weight was found to be reduced by HK1-ceramide 2, in particular of a protein with an apparent molecular weight of 116 kDa.

In contrast to HK1-ceramide 2, GM3 had to be applied in much higher concentrations of 100 µM to 500 µM to inhibit EGF receptor tyrosine phosphorylation in a dose-dependent manner (Hanai et al., 1988; Zhou et al., 1994; Rebbaa et al., 1996; Meuillet et al., 1999). We did not observe an effect at concentrations of GM3 below 100 µM. In A431 cells, tyrosine phosphorylation of the EGF receptor was found to be specifically inhibited by exogenous addition of GM3 and to a lesser extent by GM1, but not by other gangliosides or neutral glycolipids (Bremer et al., 1986). This is in line with the results of our study, where a concentration of 200 µM GM3 showed an inhibitory effect on EGF-mediated receptor tyrosine phosphorylation in A431 cells. A decrease in endogenous gangliosides in mutant Chinese hamster ovary (CHO) cells was associated with an increased autophosphorylation of EGF receptor (Weis and Davis, 1990) whereas CHO cells stably transfected with CMP-NeuAc:GM3 sialyltransferase and expressing mostly GD3 at the cell surface showed both decreased EGF receptor phosphorylation and extracellular signal-regulated kinase 2 (ERK2) activation after stimulation with EGF (Zurita et al., 2001). As expected the β-isomer HK2-ceramide 3 in concentrations in the range of 20–100 µM had no effect on EGF-dependent tyrosine phosphorylation of the EGF receptor nor of proteins with lower molecular weight in A431 cells (data not shown).

It has been argued that the lactone form of GM3 is present on tumor cells due to low pH at the surface of these cells. However, in cells exposed to low pH, GM3 had the same effect on inhibition of EGF-dependent receptor phosphorylation as in cells grown in media of neutral pH. These results show that low-pH treatment, which might promote lactonization of GM3, does not result in a further, more potent inhibition of EGF-mediated receptor phosphorylation. We could also demonstrate that low-pH treatment can activate constitutive, ligand-independent EGF receptor activity, whereas EGF-mediated tyrosine phosphorylation of the receptor is reduced under acidic conditions. In a recent study, low pH treatment has been shown to activate ERK2 MAP kinase as well as multiple MAP kinase pathways involving JNK and p38, however without increased tyrosine phosphorylation of the EGF receptor (Xue and Lucocq, 1997). In analogy to GM3 (Bremer et al., 1986) HK1-ceramide 2 has been shown to have no influence on the binding of EGF to the cell surface receptor on KB cells.

We could demonstrate here that EGF-dependent growth of KB cells, which have been characterized by the presence of high-affinity receptor and EGF-dependent mitogenesis (King and Cuatrecasas, 1982), was inhibited in a dose-dependent manner by 62%, 81%, and 86% using concentrations of 6, 17, and 25 µM HK1-ceramide 2, respectively. In contrast, the less potent GM3 required concentrations of 50 µM GM3 to inhibit EGF stimulated growth of KB cells by 65% (Bremer et al., 1986).
Our results indicate that the lactone analog of GM3, HK1-ceramide 2, is a specific inhibitor of EGF-dependent receptor tyrosine phosphorylation and consequently further signaling events to the nucleus without affecting the binding of EGF to the receptor. Surprisingly, the effect was only observed in a narrow range of inhibitor concentration. The mechanisms that has led to the inhibition on EGF signaling by HK1-ceramide 2 are currently under further investigation. The GM3 lactone analog HK1-ceramide 2 seems to be more potent in comparison to GM3 in modulation of EGF receptor activity and mitogenesis.

**Material and methods**

**Glycolipids**

HK1-ceramide 2, HK2-ceramide 3 (Tietze and Keim, 1997) and GM3 (Tomoo et al., 1996) were synthesized in the Department of Organic Chemistry of Göttingen, Germany. All glycolipids were solved in DMSO/H2O (stock solution: 2.32 mM), resulting in a final DMSO concentration of 0.86%. Human EGF was kindly provided by W. E. Schmidt (University of Bochum, Germany) and recombinant human (3-[125-I]iodotyrosyl) EGF was purchased from Amersham (Braunschweig, Germany).

**Cell culture**

Ovarial epidermoid carcinoma A431 cells and human oral epidermoid carcinoma KB cells were purchased from DSMZ (Braunschweig, Germany) (Drexler et al., 1999). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with penicillin (50 IU/ml), streptomycin (50 µg/ml), L-glutamine (2 mM) (Gibco BRL, Eggenstein, Germany), and 10% heat-inactivated fetal calf serum (FCS) (PAN, Aidenbach, Germany). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS) (PAN, Aidenbach, Germany) at 37°C in a humidified atmosphere of 5% CO2.

**Analysis of EGF-dependent tyrosine phosphorylation in A431 cells in the presence or absence of gangliosides**

Cells were seeded in 24-well plates in DMEM/10% FCS and grown to 80% confluence. The medium was replaced by DMEM without FCS and cells incubated for 24 h. Aliquots of solutions containing different quantities of HK1-ceramide 2, HK2-ceramide 3, and GM3 were added to each well as indicated and incubated for 3 h at 37°C. Cells were stimulated with EGF (50 ng/ml) 30 min prior to lysis. Addition of solvent alone (0.86% DMSO) or 1 mM ortho-vanadate (Na3VO4) 90 min prior to cell lysis were used as controls. Decreasing pH values in cell culture were produced by adding 1 N or 0.1 N HCl to the medium. pH values in medium were measured during the experiment. After washing the cells three times with cold phosphate buffered saline, the monolayers were solubilized by the addition of 125 µl lysis buffer containing 1% NP40; 0.25% Na-deoxycholate; 50 mM Tris–HCl (pH 7.4); 150 mM NaCl; 1 mM ethylene glycol bis(2-aminoethyl ether)-tetra acetic acid; 1 mM phenylmethylsulfonyl fluoride; 1 µg/ml aprotinin, leupeptin, and pepstatin A; 1 mM NaF; and 1 mM Na3VO4. The cellular lysates were centrifuged 10 min at 4°C and 21,000 × g and aliquots of the supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

Lysates were adjusted to contain equal amounts of protein, using the BCA Protein assay (Pierce, Rockford, IL).

Proteins were transferred to nitrocellulose membrane (Amersham Life Sciences, Arlington Heights, IL) and immunoblotted with antibodies, the monoclonal α–PY (4G10, IgG 2bk, 100 µg/ml, Paesel + Lorei, Duisburg, Germany), diluted 1:1000, or the polyclonal anti-EGF-R antibody (Santa Cruz Biotechnology, Heidelberg, Germany), diluted 1:1000 in 50 mM G-NET (Tris–HCl [pH 7.5]), 150 mM NaCl, 5 mM ethylenediamine tetra-acetic acid, 0.25% gelatin) overnight at 4°C. Western blots were developed using horseradish peroxidase–coupled, goat anti-mouse and goat anti-rabbit, secondary antibodies (Biorad, München, Germany), diluted 1:20,000 in 50 mM G-NET for 1 h at room temperature and enhanced chemiluminescence (Amersham). For reprobing the membrane was stripped in 70 mM Tris (pH 6.8), 2% sodium dodecyl sulfate, 0.1% β-mercaptoethanol at 55°C for 30 min.

**Determination of EGF-dependent mitogenesis**

This experiment was performed with KB cells, because mitogenic stimulation by EGF can be clearly observed in a wider range of EGF concentrations in KB cells in comparison to the results obtained in A431 cells stimulated by EGF (King and Cuatrecasas, 1982). To measure cell growth, the KB cells (1.2 × 105 cells) were seeded in 96-well plates (Nunc, GmbH & Co. KG, Wiesbaden, Germany) and cultivated in DMEM containing 5% FCS for 24 h. Then the medium was replaced with FCS-free medium, and the cells were incubated for 24 h with different quantities of gangliosides as indicated. EGF (1 ng/ml) in the presence of 100 µg/ml bovine serum albumin was added to each well and the cells were cultured for 18 h. To analyze cell proliferation the colorimetric BrdU immunoassay (Roche Diagnostics, Penzberg, Germany) was used, which is based on the measurement of BrdU incorporation during DNA synthesis.

**Analysis of binding of the 125I-EGF to cell surface receptor**

The specific binding capacity and affinity of 125I-EGF to the EGF receptor were measured in KB cells grown in 48-well plates in the presence or absence of different quantities of HK1-ceramide 2 for 24 h in DMEM without FCS. Cells were washed; 125I-EGF with a specific activity of 2.8 × 106 cpm/ng was added to each well and incubated at 4°C for 2 h. Nonspecific binding of EGF was determined by preincubation of a 500–1000-fold excess of cold EGF in four separate wells with different quantities of 125I-EGF (0.25, 0.5, 1, 3.5, 10, 15, and 20 ng/ml 125I-EGF). Cells were washed and incubated with 1 N NaOH at 25°C for 1 h, and the radioactivity associated with the cell monolayers was counted with a gamma counter (Wallac LKB1282 CompuGamma). The background value was subtracted. The effect of HK1-ceramide on the specific binding of 125I-EGF is presented by the method of Scatchard (1949).

**Acknowledgments**

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (SFB 500) and the Fonds der Chemischen Industrie.
Abbreviations
αPY, antiphosphotyrosine antibody; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; EGF, epidermal growth factor; ERK, extracellular signal-regulated kinases; FCS, fetal calf serum; MAP, mitogen-activated protein.

GM3 (NeuAcα2→3Galβ1→4Glcβ1→Cer) is abbreviated according to the nomenclature of Svensenholmen (1963), and synthesized glycolipids are abbreviated according to the International Union of Pure and Applied Chemistry (IUPAC) Nomenclature (IUPAC Commission, 1972). HK1-ceramide is (2S,3R,4E)-3-hydroxy-2-(octadecanamido)octadec-4-enyl-4-O-[3-O-(5-acetamido-1,3,5-trideoxy-D-glycero-α-D-galacto-2-nonulopyranosyl)′→2′-pyranosyl]-β-D-galactopyranosyl]-β-D-glucopyranosid. HK2-ceramide is (2S,3R,4E)-3-hydroxy-2-(octadecanamido)octadec-4-enyl-4-O-[3-O-(5-acetamido-1,3,5-trideoxy-D-glycero-β-D-galacto-2-nonulopyranosyl)′→2′-pyranosyl]-β-D-galactopyranosyl]-β-D-glucopyranosid.

References