Regulation of Epidermal Growth Factor Receptor Signal Transduction

ROLE OF GANGLIOSIDES*

Frances M. B. Weis and Roger J. Davis
From the Howard Hughes Medical Institute, Program in Molecular Medicine and Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01655

The addition of gangliosides to tissue culture cells causes a decrease in the tyrosine protein kinase activity of the epidermal growth factor (EGF) receptor and an inhibition of EGF-stimulated growth. Based on these data, the hypothesis that the EGF receptor is physiologically regulated by gangliosides has been proposed by E. G. Bremer, J. Schlessinger, and S. Hakomori (J. Biol. Chem. (1986) 261, 2434-2440). To test this hypothesis, a mutant Chinese hamster ovary cell line (clone idl D) that has a reversible defect in the biosynthesis of gangliosides (Kingsley, D. M., Kozarsky, K. F., Hobbie, L., and Krieger, M. (1986) Cell 44, 749-759) was investigated. The human EGF receptor cDNA was expressed in the mutant cells, and the properties of the EGF receptor were examined using cells grown under permissive and nonpermissive conditions. Changes in ganglioside expression were not observed to cause any significant alterations in the affinity or number of EGF receptors detected at the cell surface. However, decreased levels of ganglioside expression were associated with 1) increased EGF receptor autophosphorylation on tyrosine residues, and 2) increased EGF-stimulated cellular proliferation. The inverse correlation observed between the level of ganglioside expression and signal transduction by the EGF receptor is consistent with the hypothesis that the function of the EGF receptor is physiologically regulated by gangliosides.

The alterations in ganglioside metabolism observed during oncogenic transformation, the cell cycle, and density-dependent growth inhibition indicate that sphingolipid metabolism may play a role in the regulation of cell growth (1, 2). Consistent with this hypothesis are observations that sphingolipids are potent pharmacological regulators of cell growth and differentiation (1, 2). Direct addition of gangliosides to the tissue culture medium causes growth inhibition by extending the length of the G1 phase of the cell cycle (3, 4) and blocks cellular proliferation in the presence of fibroblast growth factor (5) and platelet-derived growth factor (6). The molecular basis for the effects of gangliosides on cell growth is not understood. One possible mechanism is that the effects of gangliosides are mediated by an inhibition of protein kinase C caused by lysosphingolipids (7, 8). However, evidence that sphingolipids interact directly with growth factor receptors has been reported. Recently, Bremer et al. (6, 9) observed that gangliosides inhibit the tyrosine kinase activity of the receptors for platelet-derived growth factor and epidermal growth factor (EGF). Gangliosides GM1 and GM2 were reported to inhibit autophosphorylation of the EGF receptor by 26 and 64%, respectively, when present in an in vitro assay at a concentration of 0.35 mM (9). Subsequent studies have demonstrated that sphingolipids are bimodal regulators of the EGF receptor which cause both stimulation and inhibition of the receptor tyrosine kinase activity (10-15).

Although sphingolipids are potent pharmacological regulators of the function of the EGF receptor, a significant question remains concerning whether the EGF receptor is physiologically regulated by sphingolipids. This is because the addition of exogenous gangliosides to tissue culture media increases the incorporation of gangliosides into cell membranes beyond the physiological range (6, 9). The purpose of the experiments presented here was to test the hypothesis that the expression of gangliosides at physiological levels modulates signal transduction by the EGF receptor. The strategy that we employed was to use a mutant CHO cell line that possesses a reversible defect in the biosynthesis of gangliosides. Using these cells we have investigated the effect of changes in the expression of gangliosides on the properties of the EGF receptor.

EXPERIMENTAL PROCEDURES

Materials—EGF was isolated from mouse submaxillary glands (16, 17) and iodinated as described (18). Bovine insulin was obtained from Sigma. Na125I, 125I goat anti mouse Ig antibody, [32P]phosphate, [3H]methionine, N-(9H)acetylimidazole, and [3H]thymidine were obtained from Du Pont-New England Nuclear. [γ-32P]ATP was prepared using a Gamma-precip A kit (Poumura Biotics) according to the manufacturer's directions. DMM was a product of Boehringer Mannheim. Sphingosine, GM1, and GM2 were obtained from Sigma. The plasmid pXER and the synthetic peptide T669 have been described previously (19). The monoclonal anti-phosphotyrosine antibody (PY20) was obtained from ICN. Membranes were prepared from CHO cells as described previously (20).

Transfection of CHO Cells—CHO cells expressing the idl D phenotype (21) were obtained from Dr. M. Krieger (Massachusetts Institute of Technology). The cells were transfected with the plasmid pXER using the calcium phosphate technique. After 3 h the cells were shocked with glycerol. Stable transfectants were selected with modified Eagle's medium supplemented with 5% dialyzed FBS and 0.5 μM amethopterin (Sigma). Colonies obtained were isolated using cloning rings and screened for expression of EGF receptors by measuring the cell surface binding of 125I-EGF at 0 °C.

Cell Culture—CHO cells were maintained in Ham's F-12 medium supplemented with 5% dialyzed FBS, 10% fetal bovine serum, GaINAc, N-acetylglactosamine, Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; synthetic peptide T669, NH2 Glu-Leu-Val-Gln-Pro-Leu-Thr-Pro-Ser-Glu-Glu-Ala-Pro-Asn-Gln-Glu-Leu-Leu-Arg-COOH; EGTA, [ethylenebis(oxyethylenenitriilo)] tetraacetic acid; G45, Gal[1-4]NeuAc[1-4]NeuAc[1-3]Gal[β1-4Glcβ1-; Cer, NeuAcα2-→3Galβ1-4Glcβ1-; Cer, NeuAcα2-→3Galβ1-4Glcβ1-; Cer.

*This work was supported in part by grants GM37845 and CA39240 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.
supplemented with 5% FBS. For each experiment the cells were transferred to medium containing 1% FBS for 24 h. The cells were then incubated for 48 h with medium not supplemented or supplemented with 200 μM GalNAc, 20 μM galactose, and 2 mM DMM.

Isolation of Gangliosides—CHO cells were cultured in 100-mm dishes for 48 h with 5 ml of medium supplemented with 100 μCi of [3H]acetate, and gangliosides were selectively extracted from the homogenates as described (22). The isolated gangliosides were desalted using a Sep-Pak (Millipore) cartridge (23) and analyzed by thin layer chromatography using silica gel plates (200 μm thick, 5–10-μm mean particle size, 60 Å mean pore diameter). The solvent used for chromatography was chloroform:methanol:0.025% CaCl2:2H2O (50:40:10). Lipid standards (Galβ1,3Galβ1,4GlcNAc, and Galα1,3Galβ1,4GlcNAc) were stained using orcinol:ferric chloride reagent (Sigma). Radioactive lipids were detected by fluorography (New England Nuclear) and were quantitated by liquid scintillation counting.

Analysis of 125I-EGF Binding—The analysis of the 125I-EGF binding to cell surface receptors was performed as described (24). The specific binding of 125I-EGF to isolated membranes was investigated as described (25).

Autophosphorylation of the EGF Receptor in Intact Cells—CHO cells were grown in 35-mm dishes and washed with serum-free medium. The cells were then incubated at 37°C for 30 min in 1 ml of 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2, 25 mM Heps (pH 7.4), 30 μM bovine serum albumin. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis as described (24). The state of tyrosine phosphorylation of the EGF receptors was investigated by a Western blot procedure (26) using a monoclonal anti-tyrosine phosphate antibody (PY20) and a 125I-labeled anti-mouse IgG second antibody. Radioactivity was quantitated using a γ-counter. Control experiments demonstrated that there was a linear relationship between the amount of phosphorylated receptor and the measured radioactivity in the immunoblot. This procedure can therefore be used for the quantitative analysis of EGF receptor tyrosine phosphorylation.

Autophosphorylation of the EGF Receptor in Vitro—Membranes (10–20 μg) were incubated in 40 μl of 25 mM Heps, 10 mM MgCl2 (pH 7.4) for 20 min at 22°C without and with 500 nM EGF. The incubations were then cooled to 0°C, and the phosphorylation reaction was initiated by the addition of 10 μl of [32P]ATP (100 μCi/nmol). After 2 min the reactions were terminated by the addition of 1 ml of 25 mM Heps (pH 7.8), 1.5% Triton, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.5 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 100 μM Na3VO4. EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis as described (24). The autophosphorylation of EGF receptor was examined by autoradiography of the dried gel. The incorporation of [32P]phosphate was quantitated by liquid scintillation counting.

3HThymidine Incorporation—CHO cells were grown in 16-mm wells and transferred to medium supplemented with 0.1% calf serum for 24 h. Growth factors and [3H]thymidine (1 μCi/ml) were then added to the cells. After incubation for a further 24 h, the incorporation of [3H]thymidine into acid-insoluble material was measured as described (27).

Phosphorylation of Synthetic Peptide T669—CHO cells were grown in 35-mm wells and incubated in 1 ml of 120 mM NaCl, 6 mM KCl, 1.2 mM CaCl2, 1 mM MgCl2, 25 mM Heps (pH 7.4), and 30 μM bovine serum albumin for 30 min at 37°C. The cells were incubated without and with 10 mM EGF for 10 min. The medium was aspirated, and the cells were collected by scraping in 250 μl of 25 mM Heps (pH 7.4), 5 mM EDTA, and 10 μg/ml leupeptin and were homogenized by 10 passages through a 26-gauge needle (4°C). Protein kinase assays were performed at 22°C using 5 μl of cell extract, 10 μl of synthetic peptide T669 (5 mg/ml), and 25 μl of 50 mM Heps (pH 7.4), 20 mM MgCl2. The phosphorylation reaction was initiated by the addition of 10 μl of 50 μM [γ-32P]ATP (100 μCi/nmol) and was terminated by the addition of 10 μl of 90% formic acid. The peptide was isolated from the reaction mixture by electrophoresis (4°C) for 3 h at 500 V on a 100-μm cellulose thin layer plate using 30% (v/v) formic acid as solvent. The phosphorylated peptide was identified by autoradiography, and the incorporation of radioactivity into the peptide was quantitated by liquid scintillation counting (19).

RESULTS

A mutant CHO cell line (ldl D) that lacks the enzyme α-L-fucosidase (21) was used for the study described here. These cells are deficient in the synthesis of both UDP-galactose and UDP-GalNac when maintained in tissue culture media containing glucose. As a result, ldl D cells exhibit pleiotropic defects in glycolipid biosynthesis and protein glycosylation (21). Addition of galactose and GalNac to the tissue culture medium allows the synthesis of UDP-galactose and UDP-GalNac via the “salvage pathway” (21). The mutant ldl D phenotype is therefore reversible and can be experimentally manipulated by the addition of sugars to the culture medium. Addition of GalNac is required for “O-linked” glycosylation and chondroitin sulfate proteoglycan expression (21). Galactose is required for ganglioside biosynthesis, terminal glycosylation of proteins, and heparan sulfate proteoglycan expression (21). The reversible nature of the ldl D phenotype makes these CHO cells useful as a model system for the investigation of the structure and function of proteins, proteoglycans, and glycolipids that contain galactose or GalNac. Previous studies have employed ldl D cells to study the low density lipoprotein receptor (21, 28), chorionic gonadotrophin (29), interleukin 2 receptor (30), heparan and chondroitin sulfate proteoglycans (31), and the transforming growth factor-β receptor (32).

Expression of the Human EGF Receptor in ldl D Cells—As CHO cells do not express EGF receptors, the human EGF receptor cDNA was introduced into ldl D cells by transfection using the calcium phosphate procedure. Stable clones were obtained which express the human EGF receptor. In initial experiments the effect of the ldl D phenotype on the state of glycosylation of the EGF receptor was investigated. Fig. 1 shows that the addition of GalNac to the cells did not cause any significant shift in the electrophoretic mobility of the EGF receptor. This result is consistent with previous reports indicating the absence of detectable O-linked glycosylation of the EGF receptor (33, 34). In contrast, the addition of galactose caused a decrease in the electrophoretic mobility of the receptor (Fig. 1A). The change in electrophoretic mobility caused by galactose was not observed in the presence of an inhibitor of terminal glycosylation, DMM (Fig. 1B). Together these data indicate that in the absence of galactose, ldl D cells are defective in the terminal glycosylation of the EGF receptor.

Ganglioside Expression by ldl D cells—To characterize further the ldl D phenotype of cells expressing the human EGF receptor, the effect of the addition of galactose to the tissue

FIG. 1. Expression of the human EGF receptor by ldl D CHO cells. ldl D cells transfected with the human EGF receptor cDNA were grown in 35-mm wells in the presence and absence of 200 μM GalNac, 20 μM galactose, and 2 mM DMM for 48 h. During the last 24 h of incubation the medium was supplemented with 10 μM [35S]methionine (100 μCi/ml). The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The figure presents an autoradiograph of the dried gel. Similar results were obtained in three separate experiments.
culture medium on the expression of gangliosides was investigated using cells metabolically labeled with N-[3H]acetylmannosamine. In the absence of galactose it was observed that the ldl D cells expressed a very low level of gangliosides. Addition of galactose to the tissue culture medium caused a marked increase in ganglioside expression (Fig. 2).

The loss of ganglioside expression by ldl D Cells grown without galactose has been observed previously by Kingsley et al. (21, 28). It was reported that a result of the deficiency in ganglioside expression is an increase in the level of the precursor for G\textsubscript{M}\textsubscript{3}, lactosylceramide (28). A decrease in the level of glucosylceramide was also observed when ldl D cells were grown in the absence of galactose (28).

**Effect of Incubation of ldl D Cells with DMM**—Galactose regulates both ganglioside expression and terminal glycosylation of the EGF receptor in ldl D cells. A method that can be used to distinguish experimentally between the effects of galactose on protein glycosylation and glycolipid synthesis is therefore required. One strategy that could be employed is the use of the inhibitor of protein terminal glycosylation, DMM (35). Control experiments were therefore performed to examine the effect of incubation of ldl D cells with DMM. Consistent with the action of DMM to inhibit mannosidase I (35), it was observed that DMM blocked the effect of galactose on the terminal glycosylation of the EGF receptor (Fig. 1). In contrast, DMM did not inhibit the increase in ganglioside biosynthesis caused by galactose (Fig. 2). An unexpected observation (35) was that incubation of ldl D cells with DMM caused a small increase in ganglioside expression in both the presence and absence of galactose (Fig. 2). It is possible that the inhibition of the utilization of UDP-galactose for protein glycosylation caused by DMM may account for the increased expression of gangliosides if the supply of UDP-galactose in ldl D cells is rate limiting for ganglioside expression.

In further experiments, the effect of DMM on EGF-stimulated growth was examined. A 1.9 ± 0.2-fold and a 1.8 ± 0.1-fold increase in the incorporation of [3H]thymidine was observed after EGF treatment of cells grown without and with DMM, respectively (mean ± S.D., n = 3). DMM therefore does not cause a marked inhibition of mitogenic signal transduction by the EGF receptor expressed in ldl D cells. Together, these data demonstrate that DMM can be employed to distinguish between the effects of galactose on protein glycosylation and glycolipid biosynthesis in ldl D cells.

**Analysis of the EGF Binding Isotherm**—The specific binding of 125I-EGF to the surface of ldl D cells was investigated at 4 °C. The 125I-EGF binding isotherm is presented graphically by the method of Scatchard (36) in Fig. 3. No statistically significant difference (F test, p < 0.05) was found between the fit of the binding data to a one-site and to a two-site model using the computer program LIGAND (37). We therefore take the fit of the data to a one-site model to be an adequate description of binding isotherm. Table I presents a summary of the binding constants for the one-site fit of the data obtained from the LIGAND program.

![Figure 2](http://www.jbc.org/)

**Fig. 2. Expression of gangliosides by ldl D CHO cells.** ldl D cells transfected with the human EGF receptor cDNA were cultured in 100-mm dishes for 48 h with Ham's F-12 medium supplemented with 1% FBS, N-[3H]acetylmannosamine (20 μCi/ml), and 200 μM GaINAc. The effect of the addition of 20 μM galactose and 2 mM DMM to the cells was investigated. Gangliosides were extracted from the cells and investigated by thin layer chromatography. Reference samples of G\textsubscript{M}\textsubscript{3} and G\textsubscript{M}\textsubscript{4} (Sigma) were applied to the thin layer plate and were located by staining with orcinol:ferric chloride reagent. Radioactive gangliosides were detected by fluorography (panel A). The level of ganglioside expression was quantitated by liquid scintillation counting. Panel B presents the mean (±S.D.) radioactivity associated with the ganglioside fraction determined in three separate experiments.

![Figure 3](http://www.jbc.org/)

**Fig. 3. Analysis of the 125I-EGF binding isotherm.** ldl D cells transfected with the EGF receptor cDNA were seeded in 16-mm wells. The cells were cultured for 48 h with Ham's F-12 medium supplemented with 1% FBS and 200 μM GaINAc in the absence (panel A) and presence (panel B) of 2 mM DMM. The effect of incubation of the cells in the absence (open symbols) and presence (closed symbols) of 20 μM galactose was investigated. The cell monolayers were washed with serum-free medium and incubated for 30 min at 37 °C. The cells were then rapidly cooled to 4 °C and incubated with 25 pm, 50 pm, 100 pm, 200 pm, 1 nm, 5 nm, and 10 nm 125I-EGF for 5 h. Nonspecific binding of 125I-EGF was determined in incubations containing a 500-fold excess of unlabelled EGF. The cells were washed rapidly, and the radioactivity associated with the cell monolayers was measured with a γ-counter. The specific binding of 125I-EGF is presented by the method of Scatchard (38). Similar results were observed in three separate experiments. The affinity of the 125I-EGF binding sites was estimated using the computer program LIGAND (37), and the data obtained are summarized in Table I.
The effect of incubation of ldl D cells with galactose and DMM on the EGF binding isotherm was investigated (Table I). The addition of galactose caused a 2-fold increase in the number of cell surface EGF binding sites. In control experiments using wild-type CHO cells expressing the human EGF receptor (13), no significant effect of galactose was observed on the EGF binding isotherm (data not shown). We conclude that the increase in the cell surface expression of the EGF receptor caused by galactose (Table I) is a result of the ldl D phenotype.

The increased EGF receptor expression at the cell surface caused by galactose (Table I) may be related to the effect of galactose to allow the terminal glycosylation of the receptor expressed in ldl D cells (Fig. 1). Consistent with this hypothesis is the observation that an inhibitor of terminal glycosylation, DMM, abolished the effect of galactose to increase the number of cell surface EGF receptors (Table I).

**Autophosphorylation of the EGF Receptor in Vitro—Membranes were prepared from ldl D cells grown in the presence and absence of galactose. EGF receptor number was assessed by measurement of the specific binding of ¹²⁵I-EGF to the membranes. The binding observed was 420 ± 30 fmol/mg and 670 ± 40 fmol/mg for membranes isolated from cells grown in the absence and presence of galactose, respectively (mean ± S.D., n = 3). The increased receptor number observed in membranes isolated from cells grown in media containing galactose compared with control cells is consistent with the effect of galactose to increase the cell surface expression of EGF receptors by intact cells (Table I). The autophosphorylation of the EGF receptors in the isolated membranes was investigated in incubations containing 8.0 fmol of EGF receptors. Autophosphorylation was initiated by the addition of 25 μM [γ-³²P]ATP and was terminated after 2 min at 4 °C. The EGF receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. Fig. 4 presents an autoradiograph of the dried gel. Similar results were observed in four separate experiments.

**Autophosphorylation of the EGF Receptor in Intact Cells—**

The autophosphorylation of the EGF receptor in intact cells was investigated using a Western blot procedure employing a monoclonal anti-tyrosine phosphate antibody. Incubation of ldl D cells with EGF caused a marked increase in the level of autophosphorylation of the EGF receptor on tyrosine residues (Fig. 5). The level of autophosphorylation was quantitated by excising the bands from the Western blots and measuring the associated radioactivity using a γ-counter. In order to compare the level of autophosphorylation observed for cells expressing different numbers of EGF receptors, the data obtained were normalized to the number of receptors detected by the analysis of the ¹²⁵I-EGF binding isotherm (Fig. 3). Fig. 6 shows that the addition of galactose to the tissue culture medium caused a marked decrease in the level of autophosphorylation of the EGF receptor in intact ldl D cells. A marked decrease in EGF receptor tyrosine phosphorylation caused by galactose was also observed when ldl D cells were cultured with DMM (Fig. 6B).

In control experiments using wild-type CHO cells expressing human EGF receptors (13), no significant effect of galactose on the tyrosine phosphorylation of the EGF receptor was observed (data not shown). We conclude that the observed regulation of EGF receptor tyrosine phosphorylation caused by galactose...
by galactose (Fig. 6A) is a result of the ldl D phenotype.

Signal Transduction by the EGF Receptor—Signal transduction by the EGF receptor expressed in ldl D cells was investigated by examination of the long term (>12 h) effects of EGF on cell growth. Fig. 7A shows that EGF caused an increase in the [3H]thymidine incorporation by cells grown in the presence and absence of galactose. However, the effect of EGF on the stimulation of [3H]thymidine incorporation was significantly greater for cells grown in the absence of galactose compared with cells grown in the presence of galactose. In control experiments, it was observed that galactose caused no significant inhibition of the EGF-stimulated growth of wild-type CHO cells expressing human EGF receptors (data not shown). We conclude that the observed inhibition of EGF-stimulated growth caused by galactose (Fig. 7A) is a result of the ldl D phenotype.

The inhibition of EGF-stimulated growth caused by galactose could be mediated by a change in the concentration of EGF required for mitogenic signal transduction as a result of a small decrease in the apparent affinity of the EGF receptor caused by galactose (Table I). To test this hypothesis, the effect of the incubation of ldl D cells with different concentrations of EGF was examined. Fig. 8A shows that galactose inhibited the EGF-stimulated incorporation of [3H]thymidine at all concentrations of EGF tested. We conclude that the effect of galactose on mitogenic signal transduction is not related to alterations in the affinity of the EGF receptor.

The effect of galactose on the EGF-stimulated incorporation of [3H]thymidine by ldl D cells could be mediated by a nonspecific effect of galactose on growth. In order to test this hypothesis we examined the specificity of the effect of galactose by investigating mitogenic signal transduction by the insulin receptor. Fig. 8B shows that galactose caused no significant inhibition of insulin action on the incorporation of [3H]thymidine by ldl D cells. Thus, galactose does not cause a nonspecific inhibition of the growth of ldl D cells.

The addition of galactose to the tissue culture medium attenuates the long term effects of EGF on the proliferation of ldl D cells (Fig. 8). In further experiments, the short term (<1 h) effects of EGF on the activation of a threonine protein kinase that phosphorylates the receptor at Thr<sup>689</sup> (19) were examined (Fig. 7B). No significant effect of the addition of galactose to the tissue culture medium was detected when the increase in threonine protein kinase activity caused by EGF was investigated (Fig. 7B). As galactose inhibits the tyrosine phosphorylation of the EGF receptor (Fig. 6), this result was unexpected. The data presented here suggest that the threshold of tyrosine phosphorylation required for short term signal transduction mediated by the T669 protein kinase activity is low. Furthermore, the contrast observed between the effects of galactose on the short term and long term effects of EGF (Fig. 7) indicates that these biochemical processes may not be closely related.

Transmodulation of the EGF Receptor—The effect of galactose on the transmodulation of the EGF receptor caused by phorbol ester was investigated. The purpose of these experiments was to test the hypothesis that ganglioside expression was required for EGF receptor transmodulation. It was observed that 4α-phorbol 12β-myristate 13α-acetate inhibited the binding of 125I-EGF to cells grown in the absence and presence of galactose (Fig. 9). Analysis of the 125I-EGF binding isotherm indicated that the mechanism of 4α-phorbol 12β-myristate 13α-acetate action was mediated by a decrease in the apparent affinity of the EGF receptor (data not shown). Thus, ganglioside expression is not required for EGF receptor transmodulation by phorbol ester.

It has been reported that sphingosine causes an increase in EGF binding to wild-type CHO cells expressing human EGF receptors (13). A similar increase in the binding of 125I-EGF
presence

surface antigens

sented are the mean ± SD. of the results obtained in three separate experiments.

was observed in experiments using ldl D cells grown in the absence and presence of galactose (Fig. 2). We conclude that the expression of gangliosides causes no significant alteration in the regulation of the EGF receptor by exogenous sphingosine. In further experiments, the regulation of $^{125}$I-EGF binding by GalNAc was investigated. Consistent with previous observations (9, 13), GalNAc caused no significant change in $^{125}$I-EGF binding to cells cultured with or without galactose (Fig. 2).

**DISCUSSION**

Gangliosides represent a major class of tumor-specific cell surface antigens (1, 2). The expression of gangliosides is regulated during the cell cycle, density-dependent growth inhibition, and oncogenic transformation (1, 2). However, the role of gangliosides in the control of cellular proliferation is not understood. Recently, pharmacological evidence has been presented demonstrating that gangliosides inhibit the tyrosine protein kinase activity of the EGF receptor (9). It is therefore possible that the function of the EGF receptor is modulated by endogenous gangliosides. In order to examine this hypothesis we have utilized a mutant CHO cell line (ldl D) that exhibits a reversible defect in the expression of gangliosides (21). The properties of the human EGF receptor expressed in ldl D cells grown under permissive and nonpermissive conditions were investigated in detail.

In the absence of galactose, ldl D cells are deficient in the biosynthesis of gangliosides (21). However, if galactose is included in the tissue culture medium the expression of gangliosides is similar to that observed in wild-type CHO cells (21). Incubation of ldl D cells in the presence and absence of galactose can therefore be used to modulate the level of ganglioside expression. A problem with this experimental approach is that ldl D cells also exhibit a defect in the terminal glycosylation of proteins in the absence of galactose. The addition of galactose to ldl D cells therefore causes at least two changes: 1) an increase in the level of glycosylated expression; and 2) an increase in the terminal glycosylation of proteins. As the state of glycosylation of the EGF receptor expressed in ldl D cells is regulated by galactose (Fig. 1), it is necessary to consider the effect of this action on EGF receptor function. Structural studies have indicated that the EGF receptor is co-translationally modified by the addition of $N$-linked oligosaccharide chains to yield a 100-kDa precursor (33, 34). Further modification of the oligosaccharide chains converts the precursor to a 170-kDa mature receptor (33, 34). The mature receptor contains both "complex" and "high mannosyl" oligosaccharide chains in a ratio of 2:1 (34). In the presence of swainsonine the 160-kDa EGF receptor precursor is transported to the cell surface where it binds EGF and possesses an active tyrosine protein kinase activity (33, 34). Thus, terminal glycosylation is not required for EGF receptor function.

In order to distinguish between the effects of galactose on ldl D cells which are related to glycolipid biosynthesis and protein glycosylation, we have employed an inhibitor of the terminal glycosylation of $N$-linked glycoproteins, DMM (35). In the presence of DMM, $N$-linked glycoproteins are expressed with unprocessed high mannosyl chains (35), and the addition of galactose to the tissue culture medium does not allow the terminal glycosylation of the EGF receptor (Fig. 1). Consequently, the effects of galactose on the level of ganglioside expression (Fig. 2) can be investigated in the absence of alterations to EGF receptor glycosylation by the inclusion of DMM in the tissue culture medium.

An additional defect present in ldl D cells is a deficiency in proteoglycan synthesis (31, 32). Chondroitin sulfate proteoglycan biosynthesis in ldl D cells is dependent on the addition of exogenous GalNAc (31). The design of the experiments reported here includes the use of 0.2 mM GalNAc in the tissue culture medium. The ldl D cells employed in these experiments therefore probably synthesize normal levels of chondroitin sulfate proteoglycans (31). However, heparan sulfate proteoglycan biosynthesis by ldl D cells requires the supplementation of the tissue culture medium with galactose (31). Consequently, the experimental strategy that we have employed to manipulate the level of ganglioside expression in ldl D cells probably also causes alterations in the expression of heparan sulfate proteoglycans. The effect of galactose on the...
Correlation between EGF Binding and Ganglioside Expression—The addition of galactose to ldl D cells was observed to cause an increase in the binding of \(^{125}\)I-EGF to cell surface receptors (Fig. 3). As galactose did not cause any marked change in the level of EGF receptor expression (Fig. 1), the increased binding of \(^{125}\)I-EGF could be accounted for by either an increase in the affinity of the EGF receptor or by the exposure of cryptic (e.g. intracellular) receptors at the cell surface. To distinguish between these alternative mechanisms, the \(^{125}\)I-EGF binding isotherm was examined. It was observed that galactose caused a marked increase in the number of binding sites expressed at the cell surface (Table I). This result indicates that galactose caused an increase in the number of cell surface EGF receptors. In the absence of galactose, the terminal glycosylation of the oligosaccharide chains on the EGF receptor is incomplete (Fig. 1). It is therefore possible that the mature EGF receptor is transported to the cell surface more efficiently than the incompletely glycosylated receptor present in cells grown without galactose (Fig. 1). Consistent with this hypothesis is the observation that galactose had no significant effect on the binding of \(^{125}\)I-EGF to cell surface receptors when ldl D cells were cultured with an inhibitor of terminal glycosylation, DMM (Table I).

The lack of a significant effect of galactose on EGF binding in the presence of DMM (Table I) indicates that the modulation of ganglioside expression (Fig. 2) has little effect on the ligand binding properties of the EGF receptor. This result is similar to that obtained in pharmacological studies that have demonstrated that the addition of gangliosides to cultured cells does not alter EGF binding (9).

Correlation between EGF Receptor Transmodulation and Ganglioside Expression—Incubation of cultured cells with phorbol ester causes an inhibition of the apparent affinity of the EGF receptor. This acute regulation of the EGF receptor has been termed transmodulation (for review, see Ref. 38). The mechanism by which phorbol ester causes transmodulation of EGF receptor is not understood (30–41). To investigate the role of gangliosides, we examined the effect of phorbol ester on the inhibition of the binding of \(^{125}\)I-EGF to the cell surface receptors of ldl D cells. It was observed that phorbol ester caused a marked inhibition of \(^{125}\)I-EGF binding to cells grown in the presence and absence of galactose (Fig. 9). We conclude that the expression of gangliosides is not required for phorbol ester action to cause EGF receptor transmodulation.

Correlation between EGF Receptor Tyrosine Protein Kinase Activity and Ganglioside Expression—The effect of the addition of galactose to ldl D cells on the tyrosine protein kinase activity of the EGF receptor was investigated. In preliminary experiments membranes were prepared from homogenates of ldl D cells using sucrose density gradient centrifugation. The in vitro tyrosine protein kinase activity of the EGF receptor was assessed by examination of the receptor autophosphorylation. No significant difference between the level of autophosphorylation of EGF receptors was observed for membranes isolated from cells grown in the absence and presence of galactose (Fig. 4). This result indicates that the modulation of ganglioside expression does not alter the in vitro autophosphorylation of the EGF receptor. In contrast, Bremer et al. (9) have reported that the addition of G\(_{M3}\) and G\(_{M5}\) to the EGF receptor inhibits the in vitro autophosphorylation of the receptor. The reason that the modulation of ganglioside expression by ldl D cells was not observed to have any significant effect on in vitro autophosphorylation of the EGF receptor (Fig. 4) may be related to the concentration of gangliosides required for inhibition. Bremer et al. (9) have reported that G\(_{M3}\) and G\(_{M5}\) inhibited the autophosphorylation of the EGF receptor by 26 and 64%, respectively, when present in an in vitro assay at a concentration of 0.35 mM. As gangliosides represent a minor component of the plasma membrane it is likely that the concentration of gangliosides present in isolated membranes is lower than that required to inhibit the autophosphorylation of the EGF receptor. This conclusion indicates that the direct inhibition of the EGF receptor tyrosine kinase activity caused by gangliosides observed in pharmacological studies (9) may not be relevant in vivo.

In further studies, the effect of galactose on the autophosphorylation of the EGF receptor using intact cells was investigated. The addition of galactose to ldl D cells caused a marked decrease in EGF receptor autophosphorylation (Fig. 6). The effect of galactose on the inhibition of autophosphorylation was also observed when ldl D cells were cultured in the presence of DMM. These data indicate that there is an inverse correlation between the level of ganglioside expression by ldl D cells and the autophosphorylation of the EGF receptor. The apparent association between an increased level of expression of gangliosides (Fig. 2) and a decreased level of EGF receptor autophosphorylation (Fig. 6) is consistent with previous reports that the addition of exogenous gangliosides to cells causes an inhibition of EGF receptor autophosphorylation (9). However, the lack of an effect of galactose on the in vitro autophosphorylation of the EGF receptor (Fig. 4) indicates that the inhibition of autophosphorylation observed in intact cells may not be due to the direct interaction of the EGF receptor with gangliosides. Further work is required to identify the mechanism of inhibition of EGF receptor autophosphorylation.

Previous studies have demonstrated that EGF receptor autophosphorylation can be regulated by the state of threonine phosphorylation of the receptor and by the receptor oligomeric state (38). It is possible that the inhibition of autophosphorylation observed in intact cells is related to one of these processes.

Correlation between Signal Transduction by the EGF Receptor and Ganglioside Expression—As galactose was observed to cause a decrease in the level of autophosphorylation of the EGF receptor in intact cells, it was anticipated that galactose might alter signal transduction by the EGF receptor. It was observed that the addition of galactose to ldl D cells caused a decrease in the stimulation of the incorporation of \(^{3}H\)thymidine caused by EGF (Fig. 7). Thus, an increase in the expression of endogenous gangliosides was associated with an inhibition of EGF-stimulated cellular proliferation. These data are consistent with the results of pharmacological studies in which the addition of exogenous gangliosides to cultured cells caused a decrease in EGF-stimulated cell growth (9).

Although it was observed that galactose caused an inhibition of EGF-stimulated cellular proliferation, no significant effect of galactose was observed on the action of EGF to stimulate the activity of a threonine protein kinase that phosphorylates the receptor at Thr\(^{689}\) (Fig. 7). This result indicates that galactose does not inhibit all the signaling functions of the EGF receptor and suggests that the signal threshold or signaling pathway may be distinct for different EGF-regulated processes.

Mechanism of Regulation of EGF Receptor Function—The results of the study reported here indicate that there is an
inverse correlation between the level of ganglioside expression by ldl D cells and signal transduction by the EGF receptor. However, further work is required to characterize the molecular mechanism by which galactose regulates the EGF receptor in ldl D cells. Identification of the galactose-regulated molecule(s) in ldl D cells that modulate the function of the EGF receptor will be an important goal for future research. It is possible that gangliosides form one class of regulatory molecule, and the results obtained in this study are consistent with the hypothesis that gangliosides represent an endogenous modulator of EGF receptor function (9). However, a role for other molecules is not excluded by the data presented in this report. Other examples of potential galactose-dependent regulatory molecules in ldl D cells include neutral glycolipids (28), sphingoid bases (8), and heparan sulfate proteoglycans (31).

Acknowledgments—Dr. M. Krieger and Dr. G. Merlin are thanked for providing ldl D cells and the plasmid pMMTV-EGFR, respectively. The excellent secretarial assistance of Karen Welch is greatly appreciated.

REFERENCES

1. Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733-764
2. Hakomori, S. (1984) Trends Biochem. Sci. 9, 453-456
3. Laine, R. A., and Hakomori, S. (1973) Biochim. Biophys. Res. Commun. 54, 1039-1045
4. Keenan, T. W., Schmid, E., Franke, W. W., and Wiegandt, H. (1975) Exp. Cell Res. 92, 259-270
5. Bremer, E. G., and Hakomori, S. (1982) Biochem. Biophys. Res. Commun. 106, 711-718
6. Bremer, E. G., Hakomori, S., Bowen-Pope, D. F., Raines, E., and Ross, R. (1984) J. Biol. Chem. 259, 6818-6825
7. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J Biol. Chem. 261, 19804-19819
8. Hannun, Y. A., and Bell, R. M. (1987) Science 235, 670-673
9. Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986) J. Biol. Chem. 261, 2439-2440
10. Hanai, N., Noreis, G. A., MacLeod, C., Torres-Mendez, C.-R., and Hakomori, S. (1988) J. Biol. Chem. 263, 10915-10921
11. Hanai, N., Doli, T., Noreis, G. A., and Hakomori, S. (1988) J. Biol. Chem. 263, 6296-6301
12. Davis, R. J., Gironés, N., and Faucher, M. (1988) J. Biol. Chem. 263, 5373-5379
13. Faucher, M., Gironés, G., Hannun, Y., Bell, R. M., and Davis, R. J. (1988) J. Biol. Chem. 263, 5319-5327
14. Northwood, I. C., and Davis, R. J. (1988) J. Biol. Chem. 263, 7450-7453
15. Wedgegartner, P. B., and Gill, G. N. (1988) J. Biol. Chem. 264, 11346-11353
16. Savage, C. R., Jr., and Cohen, S. (1972) J. Biol. Chem. 247, 7609-7611
17. Martzian, L. M., Larsen, B. R., Fineh, J. S., and Magon, B. E. (1982) Anal. Biochem. 125, 339-351
18. Peas, J. R., Glimmer, W., Oka, Y., Oppenheimer, C. L., and Czeck, M. P. (1983) J. Biol. Chem. 258, 7386-7394
19. Countaway, J. L., Northwood, I. C., and Davis, R. J. (1989) J. Biol. Chem. 264, 10828-10835
20. Davis, R. J., and Czech, M. P. (1985) J. Biol. Chem. 260, 2540-2551
21. Kingsley, D. M., Kozarsky, K. F., Hobbie, L., and Krieger, M. (1986) Cell 44, 749-759
22. Suzuki, K. (1965) J. Neurochem. 12, 629-638
23. Kudo, S. K., and Suzuki, A. (1981) J. Chromatogr. Biochem. Appl. 125, 339-351
24. Davis, R. J., and Czech, M. P. (1987) J. Biol. Chem. 262, 6832-6841
25. Massague, J. (1983) J. Biol. Chem. 258, 13614-13620
26. Burnette, N. W. (1981) Anal. Biochem. 107, 220-239
27. Davis, R. J., Ganong, B. R., Bell, R. M., and Czech, M. P. (1985) J. Biol. Chem. 260, 1565-1566
28. Kingsley, D. M., Kozarsky, K. F., Segal, M., and Krieger, M. (1986) J. Cell Biol. 102, 1576-1585
29. Matzuk, M. M., Krieger, M., Corless, C. L., and Boime, I. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 6334-6338
30. Kozarsky, K. F., Call, S. M., Dower, S. K., and Krieger, M. (1988) Mol. Cell. Biol. 8, 3357-3363
31. Edso, J. D., Rostand, K. S., and Weinke, J. L. (1988) Science 241, 1092-1096
32. Cheifetz, S., and Massague, J. (1989) J. Biol. Chem. 264, 12025-12028
33. Meyu, E. L. V., and Waterfield, M. D. (1984) EMBO J. 3, 531-537
34. Cummings, R. D., Soderquist, A. M., and Carpenter, G. (1985) J. Biol. Chem. 260, 11944-11952
35. Elheim, A. D. (1987) Annu. Rev. Biochem. 56, 497-534
36. Scatchard, G. (1949) Ann. N. Y. Acad. Sci. 51, 660-672
37. Munson, P. J., and Rodbard, D. (1980) Annu. Rev. Biochem. 50, 220-239
38. Schlessinger, J. (1988) Biochemistry 27, 3119-3123
39. Davis, R. J. (1988) J. Biol. Chem. 263, 9402-9409
40. Countaway, J. L., Gironés, N., and Davis, R. J. (1989) J. Biol. Chem. 264, 13642-13647
41. Countaway, J. L., McQuilkin, P., Gironés, N., and Davis, R. J. (1990) J. Biol. Chem. 265, 3407-3416
Regulation of epidermal growth factor receptor signal transduction. Role of gangliosides.
F M Weis and R J Davis

J. Biol. Chem. 1990, 265:12059-12066.