Two Alternative Mechanisms Control the Interconversion of Functional States of the Epidermal Growth Factor Receptor*

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Epidermal growth factor (EGF) and transforming growth factor α bind to a common receptor at the cell surface. Both the affinity and the tyrosine protein kinase activity of the receptor are regulated by exogenous factors, such as platelet-derived growth factor. A protein kinase C-dependent (Ca\(^{2+}\)/phospholipid-dependent enzyme) and independent regulatory mechanism have been described. The protein kinase C-dependent mechanism results in the inhibition of the affinity and tyrosine kinase activity of the EGF receptor. We describe in this report an alternative mechanism of regulation of the receptor that is mediated by sphingosine. Treatment of WI-38 human fetal lung fibroblasts with 5 \(\mu\)M sphingosine for 2 min at 37 °C caused a marked increase in the affinity of the EGF receptor. Similar results were obtained when isolated plasma membranes prepared from these cells were incubated with sphingosine. A stimulation of the EGF receptor tyrosine protein kinase activity was also observed after sphingosine-treatment of plasma membranes. Sphingosine caused a decrease in the \(K_a\) for ATP and an increase in the \(V_{max}\) for the tyrosine phosphorylation of a synthetic peptide substrate. Control experiments demonstrated that these actions of sphingosine were not secondary to the inhibition of protein kinase C. These data indicate that sphingosine causes the functional conversion of the EGF receptor into an activated state that expresses both a high affinity for EGF and an increased tyrosine kinase activity. We conclude that sphingosine is a bioactive molecule in human fibroblasts.

Analysis of binding isotherms has demonstrated that epidermal growth factor (EGF)\(^1\) and transforming growth factor α bind to a common cell surface receptor that exists in a high and low affinity state. Approximately 10% of the receptors are present in the high affinity state and 90% exhibit low affinity (Massague, 1983). Titration experiments have indicated that the mitogenic effect of EGF correlates with the occupancy of the high affinity receptors (Schechter et al., 1978; Kawamoto et al., 1985). In order to understand the mitogenic signaling mechanism(s) employed by the EGF receptor, it is therefore important to define the structural basis for the formation of the high affinity state of the receptor.

Transfection of cultured cells with the EGF receptor cDNA results in the expression of both high and low affinity sites at the cell surface (Schlessinger, 1986). However, recent reports have indicated that the high affinity binding sites for EGF can be distinguished from the low affinity sites and may therefore represent a distinct receptor species. First, monoclonal antibodies have been described that preferentially interact with one of the two classes of receptors (Kawamoto et al., 1983; Gregoriou and Rees, 1984). Second, it has been demonstrated by the technique of fluorescence photobleaching recovery that the high affinity receptors have a relatively restricted lateral mobility in the membrane, whereas the low affinity receptors have a high degree of lateral mobility (Rees et al., 1984).

Some insight into the mechanism of interconversion of the functional states of the EGF receptor has been provided by studies using activators of protein kinase C. Shoyab et al. (1979) showed that treatment of fibroblasts with tumor-promoting phorbol diesters caused an inhibition of the high affinity binding of EGF to cell surface receptors. Subsequent studies (Hunter et al., 1984, Davis and Czech, 1985a; Lin et al., 1986) established that the mechanism involves the phosphorylation of the EGF receptor at threonine 654 by protein kinase C. The desensitization of the EGF receptor caused by protein kinase C represents one mechanism by which the functional state of the receptor is regulated. However, this regulation cannot account for the presence of the high affinity state of the EGF receptor in control fibroblasts where 94% of the receptors are not phosphorylated at threonine 654 and only 10% exhibit high affinity (Davis and Czech, 1987). Furthermore, there is strong evidence that indicates the presence of a protein kinase C-independent mechanism of EGF receptor regulation in fibroblasts (Olashaw et al., 1986; Davis and Czech, 1987).

The purpose of the experiments described in this report was to investigate the regulation of the functional state of the EGF receptor by a protein kinase C-independent pathway. We show that sphingosine causes the formation of an activated state of the EGF receptor that possesses both a high affinity and a stimulated tyrosine protein kinase activity. As sphingolipids are differentiation-dependent, tumor-associated cell surface antigens (Hakomori 1981; 1984), sphingosine represents a physiologically relevant mediator of EGF receptor regulation that is independent of protein kinase C.

EXPERIMENTAL PROCEDURES

Materials—\(^{32}\)PPhosphate, \(^{32}\)P[ATP, \(^{3}H\)thymidine, and Na\(^{22}\) were from Du Pont-New England Nuclear. Sphingosine was obtained from Sigma. N-Acetylphosphosine was a gift from Dr. R. M. Bell (Duke University). Membranes were prepared from cultured cells

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as described (Davis and Czech, 1985b). EGF was prepared (Matrisian et al., 1983) and iodinated (Pession et al., 1983) as described.

**Cell Culture**—WI-38 fibroblasts were maintained in minimal essential Eagle's medium supplemented with 5% calf serum. A431 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% calf serum. The incorporation of [3H]thymidine by cells was measured as described (Davis et al., 1985).

**Treatment of Membranes and Cells with Sphingolipids**—Sphingosine and N-acetylsphingosine were prepared as a solution in ethanol. The sphingolipids were then added directly to 25 mM HEPES, 0.2% (w/v) bovine serum albumin (pH 7.4) and subsequently incubated with isolated membranes or with cultured cells. The final ethanol concentration was 0.1% (v/v). Ethanol (0.1%) was also included in control incubations.

**Binding Assays**—The binding of [125I]-EGF to membranes was measured as described (Massague, 1983). The binding of [125I]-EGF to cell surface receptors was measured by incubation of the cells at 0°C for 3 h as described (Davis et al., 1985).

**Phosphorylation of the EGF Receptor**—Autophosphorylation of the EGF receptor was studied by incubation of membranes (0.5 mg/ml) at 0°C with [γ-32P]ATP (50 μCi/nmol) in 25 mM HEPES (pH 7.4), 5 mM MgCl2, 50 μM Na3VO3, 10 μg/ml leupeptin, 0.5 mM EGTA in a final volume of 500 μl. In some assays 0.2% Triton X-100 was included in the incubation. The reaction was terminated by the addition of 900 μl of 25 mM HEPES (pH 7.4), 10 μg/ml leupeptin, 10 μM CaCl2, 50 mM NaF, 50 μM Na3VO3, 1.5% Triton X-100, 1% sodium deoxycholate, 0.1% NaN3, and 50 mM NaCl, and the EGF receptors were immunoprecipitated and electrophoresed on a polyacrylamide gel. The phosphorylated peptide was then located by autoradiography, excised, and the associated radioactivity by Cerenkov counting. Tryptic phosphopeptide mapping and phosphoamino acid analysis was performed as described previously (Davis and Czech, 1985a).

The phosphorylation of the EGF receptor by protein kinase C was performed by incubation of 5 mg of A431 membranes in 10 ml of 25 mM HEPES (pH 7.4), 10 mM MgCl2, 10 μg/ml leupeptin, 1 mM CaCl2, 50 mM NaF, 50 μM Na3VO3, 5 mM ATP, and 1 micromolar (1 unit = 1 μmol/min) of partially purified rat brain protein kinase C. After 10 min of incubation at 22°C, the membranes were isolated by centrifugation at 100,000 g for 60 min at 4°C. The supernatant was applied to a cellulose thin layer plate (20 μg of A431 membranes (0.5 mg/ml) at 0°C with [γ-32P]ATP (50 μCi/nmol) in 25 mM HEPES (pH 7.4), 5 mM MgCl2, 50 μM Na3VO3, 10 μg/ml leupeptin, 0.5 mM EGTA in a final volume of 50 μl. The reaction was terminated by the addition of 450 μl of 25 mM HEPES (pH 7.4), 10 mM MgCl2, 10 μg/ml leupeptin, 0.5 mM EGTA in a final volume of 50 μl. After 2 min and the EGF receptors were immunoprecipitated and electrophoresed on a polyacrylamide gel. The autophosphorylation of the receptor was quantitated by excising the gel slices containing the receptor and measuring the associated radioactivity by Cerenkov counting. Tryptic phosphopeptide mapping and phosphoamino acid analysis was performed as described previously (Davis and Czech, 1985a).

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The phosphorylation of the EGF receptor by protein kinase C was performed by incubation of 5 mg of A431 membranes in 10 ml of 25 mM HEPES (pH 7.4), 10 mM MgCl2, 10 μg/ml leupeptin, 1 mM CaCl2, 50 mM NaF, 50 μM Na3VO3, 5 mM ATP, and 1 micromolar (1 unit = 1 μmol/min) of partially purified rat brain protein kinase C. After 10 min of incubation at 22°C, the membranes were isolated by centrifugation at 100,000 g for 60 min at 4°C. The supernatant was applied to a cellulose thin layer plate and the EGF receptors were immunoprecipitated and electrophoresed on a polyacrylamide gel. The phosphorylated peptide was then located by autoradiography, excised, and the associated radioactivity was measured by Cerenkov counting. Tryptic phosphopeptide mapping and phosphoamino acid analysis was performed as described previously (Davis and Czech, 1985a).

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**RESULTS**

**Sphingosine Stimulates EGF Receptor Tyrosine Kinase Activity**—Incubation of membranes prepared from WI-38 fibroblasts with sphingosine caused an increase in the autophosphorylation of the EGF receptor (Fig. 1). A similar result was observed when the membranes were incubated with EGF. Treatment with sphingosine and EGF together resulted in a small additional increase in the autophosphorylation above that observed with either agent alone (Fig. 2). Examination of the effect of sphingosine concentration demonstrated a  

![FIG. 1. Regulation of the autophosphorylation of the EGF receptor by sphingosine. WI-38 membranes were incubated at 22°C with and without 0.2% Triton X-100. In some incubations sphingosine (10 μM) or EGF (100 nM) was included. After 20 min the samples were cooled to 4°C, and autophosphorylation was initiated by the addition of 20 μM [γ-32P]ATP. The reaction was terminated after 2 min and the EGF receptors were immunoprecipitated and electrophoresed on a polyacrylamide gel. Similar results were observed in five separate experiments.](#)

![FIG. 2. Time course of EGF receptor autophosphorylation. WI-38 membranes were incubated at 22°C with and without 5 μM sphingosine and 100 nM EGF. After 20 min the samples were cooled to 4°C and autophosphorylation was initiated by the addition of 20 μM [γ-32P]ATP. The reaction was terminated after different times and the EGF receptors were immunoprecipitated and electrophoresed on a polyacrylamide gel. The autophosphorylation of the receptor was quantitated by excising the gel slices containing the EGF receptor and measuring the associated radioactivity by Cerenkov counting. The variation observed in triplicate determinations was less than 5%. Similar results were obtained in three separate experiments. Symbols: □, control; ■, EGF; ○, sphingosine; ●, EGF and sphingosine.](#)  

biphasic dose response. Stimulation of autophosphorylation was maximal with 5 μM sphingosine. Higher concentrations (25 μM) were less effective (Fig. 3).

In addition to stimulating the autophosphorylation of the EGF receptor, sphingosine also increased the phosphorylation of an exogenous synthetic peptide substrate (Table I). Analysis of the kinetic parameters of the tyrosine phosphorylation demonstrated that both sphingosine and EGF caused a decrease in the Kₘ for substrate (ATP) and an increase in the Vmax compared with control EGF receptors (Table I). This result is in marked contrast to the effect of protein kinase C which caused a decrease in the Vmax of the phosphorylation reaction (Table I).

Although sphingosine caused a marked increase in EGF receptor tyrosine protein kinase activity, no effect of N-acetylsphingosine was observed (data not shown). The pharmacological specificity of sphingosine action was further examined by investigation of the effects of another cationic lipid, palmitoylcarnitine. No significant effect of palmitoyl-
quantitated by excising the gel slices containing the EGF receptor. Receptors were isolated by immunoprecipitation and polyacrylamide gel electrophoresis. The autoradiograph of these receptors was obtained by excising the gel slices containing the EGF receptor and measuring the associated radioactivity by Cerenkov counting.

EGF and sphingosine caused the phosphorylation of a similar set of receptor tryptic peptides (Fig. 3).

The stimulation of EGF receptor autophosphorylation was determined for the receptor in A431 membranes at 4 °C. EGF treatment of the membranes was performed at 22 °C using 200 nM EGF for 20 min. Sphingosine treatment was similarly performed using 5 μM sphingosine. Phosphorylation of the EGF receptor by partially purified protein kinase C was performed as described under "Experimental Procedures." The peptide substrate used was Arg-Arg-Leu-Ile-Glu-Glu-Thr-Tyr-Thr-Ala-Arg-Gly. To measure the rate constants of peptide phosphorylation, 20 μM ATP was used when the concentration of peptide was altered and 0.5 mg/ml peptide was used when the concentration of ATP was changed. The time of phosphorylation was 3 min. In control experiments phosphorylation was observed to be linear with time for up to 10 min. The results represent the mean and standard deviation of triplicate determinations obtained by curve-fitting to the Michaelis-Menten formalism. This was achieved by weighted nonlinear regression employing "robust" methods for the modification of residuals (Duggleby, 1981).

In further experiments the effect of the physical state of the EGF receptor on the action of sphingosine was investigated. Treatment of intact membranes with sphingosine resulted in a stimulation of the EGF receptor tyrosine kinase. In the presence of 0.2% Triton X-100, this action of sphingosine was not observed. Instead, sphingosine inhibited the tyrosine kinase activity (Figs. 1 and 3). This result suggests that the effect of sphingosine to stimulate the receptor kinase requires an intact membrane and does not occur when the EGF receptor is solubilized. However, this conclusion is equivocal because it has been previously shown that Triton X-100 alters the properties of the EGF receptor (Rubin and Earp, 1983). It is also possible that the lack of stimulation of the solubilized EGF receptor is because the sphingosine is sequestered in Triton X-100 micelles.

The stimulation of EGF receptor autophosphorylation was examined by phosphopeptide mapping. It was observed that EGF and sphingosine caused the phosphorylation of a similar set of receptor tryptic peptides (Fig. 4). Phosphoamino acid analysis demonstrated the presence of [32P]phosphothreonine in all of the peptide fractions detected during the HPLC analysis. This result demonstrates that both EGF and sphingosine caused the autophosphorylation of the receptor on tyrosine residues. In addition to the presence of [32P]phosphothreonine, it was observed that one peptide (5 eluting at 27% acetonitrile from the reverse-phase column contained [32P]phosphothreonine. This threonine phosphorylation of the EGF receptor was observed after treatment of membranes with EGF or with sphingosine. In four experiments it was found that sphingosine (10 μM) caused a greater extent of threonine phosphorylation of the EGF receptor than that observed after EGF (100 nM) treatment (Fig. 4).

**Sphingosine Increases the Affinity of the EGF Receptor**—Treatment of WI-38 fibroblast membranes with sphingosine caused an increase in the specific binding of 125I-EGF to receptors (Fig. 5). Maximal effects of sphingosine were observed at a concentration of approximately 5 μM. No effect of N-acetylsphingosine was observed on the binding of 125I-EGF to membranes (Fig. 5). Scatchard analysis of the binding of 125I-EGF to the fibroblast membranes demonstrated the presence of a large population of low affinity (Kd = 9 × 10⁻¹⁰ M) and a small population of high affinity (Kd = 8 × 10⁻¹¹ M) receptors (Fig. 5). After treatment of the membranes with sphingosine, a linear Scatchard plot was obtained indicating the presence of a single class of high affinity (Kd = 1 × 10⁻¹⁰ M) receptors.

**Sphingosine Regulates the EGF Receptor in Intact Human Fibroblasts**—Sphingosine potently regulates the function of the EGF receptor in vitro. It was therefore important to investigate whether sphingosine was capable of regulating the EGF receptor in vivo. Treatment of intact WI-38 fibroblasts with sphingosine caused a marked increase in the binding of 125I-EGF to cell surface receptors. Scatchard analysis demonstrated that the increase in the binding of 125I-EGF was the result of an increase in the affinity of the EGF receptors (Fig. 6) similar to that observed in vitro (Fig. 5). The time course of the effect of sphingosine to regulate the apparent affinity of the EGF receptor was investigated. An increase in the binding of 125I-EGF was observed within 2 min of treatment.
**Fig. 4. Analysis of EGF receptor phosphorylation by HPLC tryptic phosphopeptide mapping.** Autophosphorylated EGF receptors were prepared as described in the legend to Fig. 1. The isolated receptors were then digested with trypsin and the resulting mixture of [32P]phosphopeptides were resolved by reverse-phase HPLC. The profile observed during the elution of the column with a gradient of acetonitrile is presented in panel A. Six phosphopeptide fractions were identified and the results of phosphoamino acid analysis are presented in panel B. Similar results were obtained in three separate experiments.

Recently, it has been reported that sphingosine is a potent inhibitor of protein kinase C (Hannun et al., 1986). It is therefore possible that the increase in the affinity of the EGF receptor caused by sphingosine is secondary to the inhibition of protein kinase C. Consistent with this proposal, it was observed that the inhibition of [125I]-EGF binding to fibroblasts caused by activating protein kinase C with phorbol ester could be partially inhibited by treatment of the cells with sphingosine (Table IV). To test the hypothesis that the effect of sphingosine could be accounted for by the inhibition of protein kinase C, the effect of sphingosine on control and protein kinase C-deficient fibroblasts was examined. It was observed that sphingosine stimulated the binding of [125I]-EGF to both control fibroblasts and fibroblasts incubated with a high concentration of phorbol ester to down-regulate protein kinase C (Table IV).

As sphingosine has been shown to be cytotoxic for some cells (Merrill, 1983; Merrill et al., 1986) the effect of sphingosine on the growth of WI-38 fibroblasts was examined (Fig. 6). Exponentially growing fibroblasts were incubated with different concentrations of sphingosine and the incorporation of [3H]thymidine by the cells was measured after 24 h. Low concentrations of sphingosine (1–5 μM) that regulate the EGF
concentrations of sphingosine for 24 h was measured. Similar results were obtained in two separate experiments. EGF for 3 h. After washing the cell monolayers, the bound \(^1\text{H}-\text{EGF}\) was measured using a gamma counter. Nonspecific binding was estimated in incubations containing 200 nM EGF. The results are expressed as the mean and standard deviation of triplicate determinations.

**TABLE II**
Effect of temperature on the regulation of the apparent affinity of the EGF receptor by sphingosine

| Temperature (°C) | Binding of \(^{125}\text{I}-\text{EGF}\) in Control | Sphingosine (10 \(\mu\text{M}\)) |
|------------------|-----------------------------|-----------------------------|
| 4                | 989 ± 24                    | 986 ± 11                    |
| 22               | 1007 ± 25                   | 2652 ± 106                  |
| 37               | 1016 ± 27                   | 2234 ± 191                  |

**Fig. 6.** Regulation of the apparent affinity of the EGF receptor in intact human fibroblasts by sphingolipids. A, WI-38 fibroblasts were incubated for 5 min at 37°C with different concentrations of sphingosine (4) or N-acetylphosphosine (C). The cells were then rapidly cooled to 0°C and the binding of 200 pM \(^{125}\text{I}-\text{EGF}\) to cell surface receptors was determined after incubation for 3 h. Nonspecific binding was determined in incubations with 200 nM EGF. The specific binding of \(^{125}\text{I}-\text{EGF}\) to the cell surface receptors of WI-38 fibroblasts is presented as the mean of triplicate determinations. The variation in the data was less than 10%. Similar results were obtained in four experiments. In a separate experiment the incorporation of \(^{3}\text{H}\)thymidine by WI-38 fibroblasts incubated with 200 nM \(^{125}\text{I}-\text{EGF}\) to cell surface receptors of WI-38 fibroblasts was presented as the mean of triplicate determinations. The variation in the data was less than 10%. Similar results were obtained in two separate experiments.

**TABLE III**
Effect of sphingosine on the phosphorylation state of the EGF receptor in intact fibroblasts

|                        | \(^{3}\text{P}\)Phosphosine | \(^{3}\text{P}\)Phosphothreonine | \(^{3}\text{P}\)Phosphyotyrosine |
|------------------------|-----------------------------|-------------------------------|-------------------------------|
| Control                | 380 ± 31                    | 560 ± 57                      | 620 ± 90                      |
| Sphingosine            | 115 ± 23                    | 340 ± 32                      | 310 ± 28                      |
| Sphingosine and EGF    | 15 ± 4                      | 85 ± 21                       | 42 ± 17                       |

**TABLE IV**
Regulation of the apparent affinity of the EGF receptor in control and protein kinase C-deficient fibroblasts

|                        | Control 565 ± 27 | Sphingosine 2764 ± 99 | PMA 301 ± 3 | Sphingosine and PMA 1916 ± 66 |
|------------------------|-----------------|-----------------------|-------------|-----------------------------|

The results presented represent the mean and standard deviation of the radioactivity recovered as \(^{3}\text{P}\)phosphoserine, \(^{3}\text{P}\)phosphothreonine, and \(^{3}\text{P}\)phosphotyrosine in three determinations.

**DISCUSSION**

Functional Activity of the EGF Receptor Is Regulated by Two Alternative Pathways—In this report we demonstrate that sphingosine causes an increase in the affinity and tyrosine protein kinase activity of the EGF receptor. An alternative pathway of EGF receptor regulation is provided by protein kinase C which causes the inhibition of the high affinity binding of EGF to the receptor and inhibits the tyrosine receptor affinity (Figs. 5 and 6) and tyrosine kinase activity (Fig. 3) did not change the incorporation of \(^{3}\text{H}\)thymidine by the fibroblasts. Sphingosine was not observed to stimulate the growth of the fibroblasts (Fig. 6). This result indicates that the stimulation of tyrosine phosphorylation by sphingosine (Table III) is not associated with growth. This may be due to a failure of sphingosine to mimic other actions of EGF that are required for stimulated growth or may be due to additional actions of sphingosine that are toxic. High concentrations of sphingosine (>25 \(\mu\text{M}\)) were strongly cytotoxic (Fig. 6). These data demonstrate that the regulation of the EGF receptor by sphingosine in intact cells is not secondary to its cytotoxic action which is only observed at high concentrations.
protein kinase activity (for review see Davis and Czech, 1985c). The regulation of the functional state of the EGF receptor by these alternative mechanisms is presented schematically in Fig. 7.

As sphingosine and lysosphingolipids have been reported to be potent inhibitors of protein kinase C (Hannun et al., 1986; Hannun and Bell, 1987) it is possible that the activation of the functional activity of the EGF receptor caused by sphingosine is secondary to the inhibition of protein kinase C. However, several lines of evidence indicate that the primary action of sphingosine to regulate the EGF receptor is independent of protein kinase C. First, sphingosine causes a marked increase in the affinity of EGF receptors in human fibroblasts (Fig. 6) under conditions where control cells exhibit a stoichiometry of threonine 654 phosphorylation of only 6% (Davis and Czech, 1987). An inhibition of this low stoichiometry of phosphorylation caused by sphingosine is unlikely to account for the change in the affinity of the EGF receptor observed (Fig. 6). Second, in vitro experiments demonstrate that sphingosine activates the tyrosine protein kinase activity of the EGF receptor in the absence of any phosphorylation of threonine 654 as demonstrated by phosphopeptide mapping (Fig. 4). Third, the effect of sphingosine to cause an increase in the affinity of the EGF receptor in vitro is observed in the absence of ATP (Fig. 5), and in vivo it is observed in the absence of protein kinase C (Table III). We conclude that the regulation of the functional state of the EGF receptor caused by sphingosine and protein kinase C are mediated by two separate mechanisms (Fig. 7).

What Is the Molecular Basis for the Interconversion of the Functional States of the EGF Receptor?—The regulation of the functional state of the EGF receptor by sphingosine may be caused by the direct or indirect interaction of sphingosine with the EGF receptor. An example of an indirect action of sphingosine is the possible role of lipid-phase catalysis of EGF binding to the EGF receptor (Sargent and Schwyzer, 1986). Alternatively, sphingosine may perturb the lipid bilayer resulting in a change in the functional properties of the EGF receptor. A direct interaction of sphingosine with the EGF receptor is not excluded by the data presented in this report.

Yarden and Schlessinger (1987a, 1987b) have proposed that the EGF receptor tyrosine protein kinase activity is stimulated by an EGF-dependent allosteric oligomerization of the receptor (but see Basu et al., 1986). At a molecular level it is proposed that the EGF receptor is in rapid equilibrium between an inactive monomeric and an active dimeric state. According to this model EGF stimulates the tyrosine protein kinase activity of the receptor by converting the EGF receptor into the dimeric state. The regulation of the EGF receptor by the two alternative pathways described in this report (Fig. 7) can be interpreted within the framework of the allosteric oligomerization model of EGF receptor functional regulation (Schlessinger, 1986). Thus, it is possible that the mechanism of sphingosine action is to stabilize the dimeric state of the EGF receptor and that the phosphorylation of the receptor by protein kinase C stabilizes the monomeric state of the EGF receptor.

It is of interest that recently the activated state of two other receptors has been proposed to involve the aggregation of receptor molecules. First, it has been shown that the high affinity state of the insulin receptor is due to the association of two ligand-binding domains (Boni-Schnetzler et al., 1987; Sweet et al., 1987). Second, in the case of the interleukin 2 receptor, the high affinity state has been demonstrated to be due to the association of the Tac protein with anotherprotein subunit of the receptor (Sharon et al., 1986; Tsudo et al., 1986; Teshigawara et al., 1987, Robb et al., 1987).

Regulation of EGF Receptor Function by Sphingolipids—Relatively little is known about the functions of sphingolipids. It has been shown that gangliosides act as the receptors for bacterial toxins (Fishman, 1982) and viruses (Markwell et al., 1981). Furthermore, gangliosides represent a major class of tumor-specific cell surface antigens (Hakomori, 1981, 1984). In addition, 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 (Hakomori, 1981, 1984). More recently several reports have suggested a direct effect of sphingolipids on the control of cell growth. First, exogenous gangliosides are mitogenic for astroglial cells (Katoh-Semba, et al., 1986) and neuroblastoma cells (Tsuji et al., 1983) but are growth inhibitory for murine 3T3 fibroblasts (Bremer et al., 1984). Second, the B-subunit of cholera toxin, which binds to ganglioside GM1, is mitogenic for murine 3T3 fibroblasts (Spiegel and Fishman, 1987). Third, it has been demonstrated that sphingosine is a potent inhibitor of protein kinase C (Hannun et al., 1986). This information strongly suggests that sphingolipids are physiologically relevant regulatory biomolecules.

Bremer et al. (1984, 1986) have reported that gangliosides inhibit the tyrosine protein kinase activities of the receptors for platelet-derived growth factor and EGF. Gangliosides Gm1 and Gm2 were reported to inhibit the autophosphorylation of the EGF receptor by 26 and 64%, respectively, when present at a concentration of 0.35 mM (Bremer et al., 1986). This inhibition of receptor tyrosine kinases by gangliosides may be physiologically relevant, but it occurs at a concentration that is significantly higher than that reported here for the action of sphingosine to activate the EGF receptor. At a concentration of sphingosine of 5 mM, the EGF receptor affinity and tyrosine kinase activity is maximally stimulated (Figs. 3 and 5). Treatment with 5 mM Gm1, Gm2, or Gm3 under similar conditions caused no significant change in the binding of 125I-EGF to the receptor or the tyrosine protein kinase activity.2

Although sphingosine is a potent pharmacological regulator of EGF receptor function, a significant question remains concerning whether the EGF receptor is physiologically regulated by sphingosine or by other sphingolipids. The major

![Fig. 7. Schematic representation of the regulation of the functional state of the EGF receptor by sphingolipids and protein kinase C. Two pathways of EGF receptor regulation are identified. First, sphingosine causes an increase in the affinity of the receptor and an activation of the tyrosine kinase activity. Second, protein kinase C phosphorylates the EGF receptor at threonine 654 and causes an inhibition of high affinity binding of EGF and inhibits the tyrosine kinase activity.](image)
pools of sphingolipid in cells are present as sphingomyelin, ceramides, and gangliosides. Sphingosine/lyso sphingolipids are probably derived by sphingolipid breakdown (Merrill and Wang, 1986). The level of free long-chain sphingoid bases in pools of sphingolipid in cells are present as sphingomyelin, the major classes of sphingolipids. Recently, Hanai et al. (1986) have measured the level of free sphingosine in HL60 human promyelocytic leukemia cells to be 12 pmol/10^6 cells. This level of sphingosine is considerably in excess of that observed in HL60 cells (Merrill et al., 1986). However, this calculation depends upon two major assumptions. First, that all sphingosine added to cultured cells occurs. Second, that all of the sphingosine added to the medium is accumulated by the cells into the correct physiological compartment. As the medium contains albumin, which will bind hydrophobic ligands such as sphingosine, it is unlikely that all of the sphingosine is effectively delivered to the cells. Evidence to support this proposal has recently been reported by Merrill et al. (1986) in studies designed to investigate the time course of accumulation of [3H]dihydrosphingosine by HL60 promyelocytic leukemia cells. The half-time of accumulation was measured to be 6 h. This slow rate of accumulation is in marked contrast to the rapid accumulation of 5 μM sphingosine to increase the affinity of the EGF receptor within 2 min of treatment of WI-38 fibroblasts at 37°C. These data indicate that the effective intracellular level of sphingosine required for the activation of the EGF receptor within WI-38 fibroblasts is probably significantly lower than our estimate of 10 nmol/10^6 cells.

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