Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1893–1897, March 1982
Cell Biology

Cell surface insertion of exogenous epidermal growth factor receptors into receptor$^-$ mutant cells: Demonstration of insertion in the absence of added fusogenic agents

(DNA synthesis/cell division/protein transfer during cell–cell contact)

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Communicated by Harry Harris, December 10, 1981

ABSTRACT **We show that epidermal growth factor (EGF) receptor can be transferred in a biologically active orientation from donor hepatic membranes to recipient receptorless fibroblast cells. The recipient cells (NR-6) normally lack EGF receptors and are biologically unresponsive to EGF. The transfer of receptors from donor plasma membranes to recipient NR-6 surface membranes occurs in the absence of any added fusogenic agent. Studies on time and temperature dependence of this transfer indicate that it is due to preferential insertion of the EGF receptor over the other hepatic proteins. The inserted receptor is exceptionally stable to dissociation or damage, and this facilitated studies on its biological properties. The inserted receptor confers upon the hitherto unresponsive variant NR-6 cells a specific biological responsiveness to EGF as measured by EGF-induced stimulation of DNA replication and cell division. These findings suggest the existence of an affinity-mediated mechanism for the biologically active insertion of exogenous EGF receptors into receptorless variant cells. This insertion approach may be of use in the identification of receptor-associated membrane proteins that play a role in the transmission of EGF biological message.**

A cell's ability to recognize and respond to changes in the outer environment is mediated through a series of externally oriented receptors or recognition molecules. One way to obtain insights into the mechanism of receptor action would be to induce a cell to insert into its plasma membrane a receptor that it normally lacks. Polyethylene glycol-mediated membrane fusion techniques have been used for putting foreign receptors into recipient cells and for combining components from two different types of cells for studies on coupling between hormone–receptor complexes and adenylate cyclase (1–3). The present paper demonstrates that an exogenous epidermal growth factor (EGF) receptor can be inserted into a recipient cell (in a biologically active orientation) by a physiological mechanism requiring no added fusogenic agent.

EGF, a single-chain mitogenic polypeptide of M_r 6045, interacts with responsive cells through high-affinity surface receptors (4). The membrane receptor for EGF in mouse 3T3 cells is a polypeptide of $M_r \approx 180{,}000$, and interaction of EGF with this surface receptor has been shown to lead to the intracellular generation of a macromolecular activator of DNA replication (5–8). We were interested in extending these studies to a variant cell line, NR-6 derived from mouse 3T3, which can neither bind nor biologically respond to EGF (9, 10). Development of a procedure for insertion of exogenous EGF receptor into the plasma membrane of these NR-6 cells could be an important step toward elucidation of the mechanism of receptor action and understanding the mode of integration of receptor proteins in the membrane.

In this communication we report that EGF receptor from purified mouse liver plasma membranes is transferred to NR-6 cell surface membrane by a mechanism requiring no added fusogenic agent. Preliminary time- and temperature-dependence studies indicate that most of this transfer is due to a preferential insertion of the EGF receptor over the bulk hepatic proteins. The inserted receptor binds to EGF with high specificity and confers upon the normally EGF unresponsive NR-6 cells a specific biological responsiveness to EGF as measured by stimulation of DNA replication and cell division.

MATERIALS AND METHODS

Materials. [^{3}H]Thymidine was a product of New England Nuclear. Calf serum (fetal and newborn) was from GIBCO. Murine EGF was prepared by using the procedure of Savage and Cohen (11). ^{125}I-Labeled EGF (^{125}I-EGF) (specific radioactivity, 1–2 × 10^5 cpm/ng) was prepared as described (10).

Cell Culture. Monolayer cultures of mouse NR-6 cells (a kind gift from Harvey Herschman) were grown and maintained at 37°C in a 10% CO_2/90% air atmosphere in Dulbecco's modified Eagle's medium containing 3% fetal calf serum and 7% newborn calf serum. Cells were subcultured by trypsinization. Routine tests showed the cells to be free of mycoplasma contamination.

Preparation of a Plasma Membrane Fraction from Mouse Liver. The procedure of Aronson and Touster (12) for isolation of rat liver plasma membranes was used without any modification for preparation of liver plasma membranes from Swiss white mice. The 33,000 × *g*–78,000 × *g* pellet was suspended in 57% sucrose/5 mM Tris·HCl, pH 8.0, and subjected to sucrose gradient equilibrium centrifugation as described. The fraction P_2 (8.5–34% sucrose) contained about 25% of the input 5′-nucleotidase activity and EGF binding activity and less than 0.1% of the input *N*-acetyl-β-D-glucosaminidase activity present in the original tissue homogenate. The specific EGF-binding activity of this fraction varied between 1 and 3 pmol of ^{125}I-EGF bound per mg of protein under standard incubation conditions (60-min incubation with 10 nM ^{125}I-EGF at 20°C). This fraction, referred to as mouse liver membranes, was used for the receptor insertion experiments described in this paper.

EGF Receptor Insertion. NR-6 cells treated with liver membrane in 16-mm dishes were washed free of unbound membranes and tested for surface EGF receptor activity as follows. The cells were incubated at 20°C for 60 min with 10 nM ^{125}I-EGF in Earle's balanced salt solution containing 10 mM Hepes (pH 7.4) and 0.1% bovine serum albumin. At the end of incubation, unbound radioactivity was removed by rapidly washing

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Abbreviation: EGF, epidermal growth factor.

the cell monolayers five times with 1 ml of the same salt/albumin solution for each wash. The washed monolayers were solubilized with 0.5 ml of 0.5 M NaOH and assayed in a gamma counter. Specific binding was determined by measuring the difference in cell-bound radioactivity in the presence and absence of 2 μg of unlabeled EGF.

Radioiodination of Mouse Liver Membranes. Membranes (1–3 mg of protein, in 250 μl of 0.15 M NaCl/10 mM Tris·HCl, pH 7.4) were labeled with ^{125}I (0.1–0.5 mCi; 1 Ci = 3.7×10^{10} becquerels; Amersham) at 0°C for 10 min with IODO-GEN (50–100 μg; Pierce) as the oxidizing agent (13). The radioactivity labeled membranes were pelleted by high-speed centrifugation, washed twice with 0.15 M NaCl/10 mM Tris·HCl, pH 7.4, and used for assay of cellular insertion of bulk hepatic membrane proteins. Electrophoresis and autoradiography showed that almost all the Coomassie blue-stained protein bands were labeled with ^{125}I.

Bulk Hepatic Protein Insertion. NR-6 cells that had been treated with ^{125}I-labeled mouse liver membranes in 16-mm dishes were washed free of unbound membranes. The washed cells containing bound labeled membranes were solubilized with 0.5 ml of 0.5 M NaOH and assayed in a gamma counter.

DNA Synthesis. Cell monolayers in 16-mm dishes (treated with membranes and EGF as described in the text) were incubated at 37°C with [^{3}H]thymidine (1 μCi/ml; 0.65 μM) in 0.5 ml of conditioned Dulbecco's modified Eagle's medium containing 2% fetal calf serum. At the end of incubation (1 or 12 hr), the trichloroacetic acid-insoluble radioactivity was determined as described (5).

Protein Determination. Protein was determined by using the procedure of Lowry *et al.* (14), with bovine serum albumin as the standard.

RESULTS

Time and Temperature Dependence of Receptor Insertion. When hepatic membranes were incubated with NR-6 cells at 26°C for 2 hr and then tested for insertion, only about 1–3% of the input EGF receptor and input bulk membrane proteins were transferred from membranes to NR-6 cells (Table 1). However, when the incubation time was prolonged up to 6 hr there was a transfer of almost 20% of input receptor activity whereas bulk protein transfer remained near the original 1% level. Receptor insertion after 10 or 12 hr of incubation at 26°C was about the same as that observed after 6 hr of incubation, suggesting attainment of equilibrium within 6 hr. The results suggest that, during the earlier part of the incubation (2 hr), bulk membrane adhesion occurred but later a specific mechanism took control causing a preferential insertion of the EGF receptor over the other hepatic proteins. Preincubation of NR-6 cells with cycloheximide (1 μg/ml) or the presence of cycloheximide (1 μg/ml) during incubation of cells with hepatic membranes had no effect on the receptor gain by the NR-6 cells, suggesting that endogenous protein synthesis is not involved in this process (data not shown). Also, the process does not involve glycosylation-induced activation of preexisting aglyco receptors in NR-6 cells because tunicamycin (1 μg/ml) did not inhibit the receptor gain (data not shown).

Table 1. Time dependence of receptor insertion

Incubation, hr	% input inserted into NR-6 cells	
	EGF receptor*	Hepatic protein†
2	3.0	1.0
4	9.0	1.2
6	19.0	1.5
10	20.5	1.5
12	19.0	1.7

The results shown represent the average of two separate experiments.

* Monolayers of NR-6 cells in 16-mm dishes were incubated at 26°C for the indicated time periods with mouse liver membranes (140 μg of protein) in 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum. At the end of incubation, the cells were tested for surface inserted EGF-receptor activity. In these experiments, 100% input receptor activity represented binding of 120 fmol of ^{125}I-EGF (160,000 cpm) at 10 nM ^{125}I-EGF.

† Monolayers of NR-6 cells in 16-mm dishes were incubated at 26°C for the indicated time periods with ^{125}I-labeled mouse liver membranes (140 μg of protein) in 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum. At the end of incubation, the cells were tested for bulk hepatic protein insertion. In these experiments, 100% input bulk hepatic membrane proteins represented 3,000,000 cpm of ^{125}I radioactivity.

A preferential insertion of the EGF receptor was also observed in a temperature-dependence study (Fig. 1). Even at 4°C, at which there was insertion of only 0.7% of the input bulk membrane proteins, EGF-receptor insertion was up to 7% of the input activity. At 24°C, approximately 19% of the input EGF receptors were inserted, whereas only about 1% of the bulk membrane proteins were inserted. At 37°C, receptor insertion increased, but there was also an increase in bulk membrane incorporation, probably due to an enhanced rate of endocytic uptake. Because the mechanism responsible for preferential receptor insertion operated optimally at 24–26°C, with a low degree of nonspecific membrane incorporation, this temperature range was used in all the subsequent insertion experiments

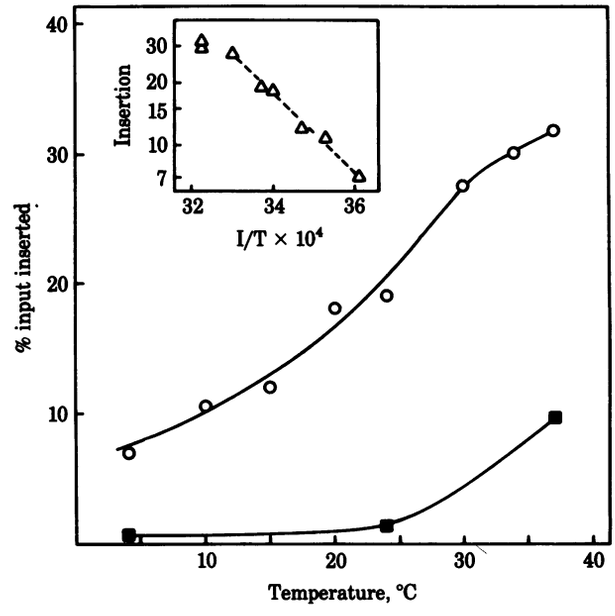

FIG. 1. Temperature dependence of receptor insertion. For determination of EGF-receptor insertion (○), monolayers of NR-6 cells in 16-mm dishes were incubated with 140 μg of mouse liver membranes in 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum for 6 hr at the indicated temperatures. At the end of incubation, inserted EGF-receptor activity was measured. In this experiment, 100% input receptor activity represented binding of 105 fmol of ^{125}I-EGF (150,000 cpm) at 10 nM ^{125}I-EGF. For determination of bulk membrane protein insertion (■), monolayers of NR-6 cells in 16-mm dishes were incubated with 140 μg of ^{125}I-labeled mouse liver membranes (58,000 cpm/μg of protein) in 0.3 ml of the same medium for 6 hr at the indicated temperatures. At the end of incubation, bulk hepatic protein insertion was measured. (*Inset*) Arrhenius plot of the EGF-receptor insertion data (% of receptors inserted plotted against reciprocal of absolute temperature). The results shown here represent the average of two separate experiments.

described in this paper. Fig. 1 (*Inset*) shows an Arrhenius plot of the temperature-dependence data. The average activation energy for receptor insertion in the temperature range of 4–30°C was approximately 8.8 kcal (1 cal = 4.18 J).

Characteristics of the Inserted Receptor. The inserted receptor could bind ^{125}I-EGF with high specificity and affinity (Fig. 2). The apparent dissociation constant of the binding of ^{125}I-EGF with the inserted receptor was about 2.4 nM, in agreement with that observed (2.2 nM) for binding of ^{125}I-EGF to the donor hepatic receptor in isolated plasma membranes. A large fraction (90%) of the ^{125}I-EGF bound at 23°C to NR-6 cells containing inserted receptors could be dissociated from the cells by mild acid treatment (0.2 M acetic acid/0.5 M NaCl, pH 2.5, 6 min at 4°C), suggesting a surface location for the inserted receptors.

The number of EGF receptors that could be inserted into an NR-6 cell during a 6-hr incubation at 26°C increased in proportion to quantity of donor membranes and then gradually plateaued (Fig. 3). At maximal insertion, approximately 10^6 receptor sites were incorporated per NR-6 cell. The parent 3T3 cell line (from which the NR-6 line was derived) contains approximately 10^5 EGF-receptor sites per cell (10).

Pretreatment of the EGF receptors in the donor membranes with a photoreactive derivative of ^{125}I-EGF resulted in specific radiolabeling of the receptor due to the formation of a M_r 170,000 receptor–^{125}I-EGF covalent complex (10). Transfer of this covalent complex to NR-6 cells also increased in proportion to the quantity of donor membranes (Fig. 3). This provided an extra criterion for true transfer of receptor from donor membranes to recipient cells.

Stability of the Inserted Receptor. The inserted receptor was exceptionally stable to dissociation and other losses (Fig. 4). After an initial loss of about 40% of the original inserted activity between 10 and 20 hr at 37°C, there was no further loss of receptor up to at least 50 hr of incubation at 37°C. A parallel experiment conducted with ^{125}I-labeled hepatic membranes showed a rapid loss (80% lost within 5 hr) of bulk inserted proteins (data not shown).

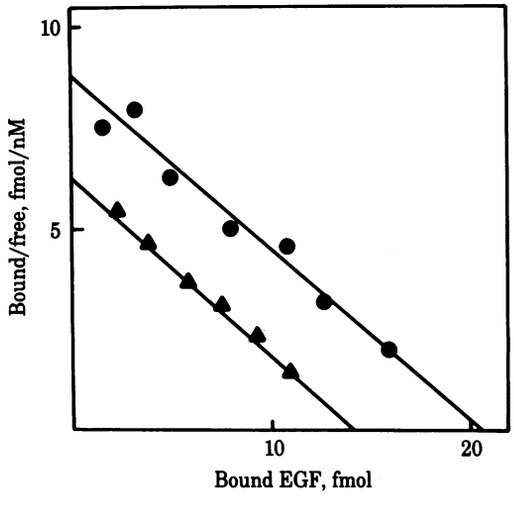

FIG. 2. Scatchard plot of ^{125}I-EGF binding to the inserted receptor. Monolayers of NR-6 cells in 16-mm dishes were incubated for 6 hr with 90 μg of hepatic membrane proteins as described in the legend for Table 1. The membrane-treated cells were incubated at 20°C for 1 hr with ^{125}I-EGF (190,000 cpm/ng) at 0.2, 0.4, 0.8, 1.6, 2.4, 4, and 8 nM and tested for specific EGF-binding activity (●). Nonspecific binding for the membrane-treated NR-6 cells at 0.42, 1.6, and 4 nM ^{125}I-EGF was 0.045, 0.18, and 0.48 fmol, respectively. NR-6 cells not treated with membranes showed no specific binding. EGF binding activity of the donor hepatic membranes (▲) was measured by incubating 10 μg of membranes at 20°C for 60 min with ^{125}I-EGF at 0.2, 0.4, 0.8, 1.6, 2.4, 4, and 8 nM. At the end of incubation, membrane-bound ^{125}I-EGF was separated from unbound ^{125}I-EGF by filtration on Millipore EHWP filters. The data shown here represent the average of three separate experiments.

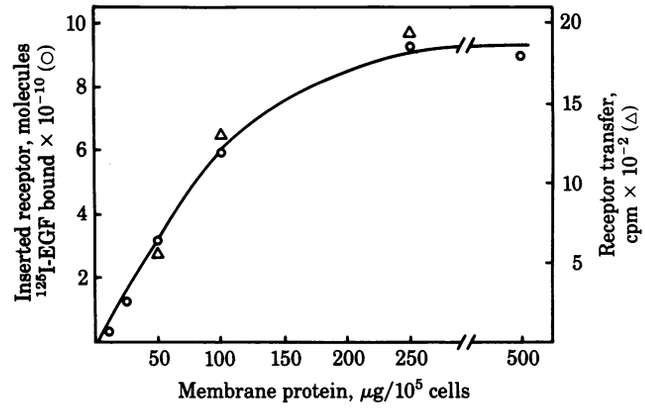

FIG. 3. Dependence of receptor insertion on membrane concentration. Monolayers of NR-6 cells in 16-mm dishes were incubated with the indicated amounts of mouse liver membrane proteins in 0.25 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum at 26°C for 6 hr. At the end of incubation, the monolayers were tested for EGF-receptor insertion (○). In other experiments, the donor membranes were pretreated with photoreactive ^{125}I-EGF so that the EGF receptor was specifically radiolabeled (10). Transfer of the radiolabeled hepatic EGF receptor to the NR-6 cells was measured after electrophoresis and autoradiography by measuring the radioactivity present in the receptor band (△).

Inserted Receptor and Stimulation of DNA Replication and Cell Division. In order to determine whether the inserted receptor behaved like a normal functional receptor, we tested it for EGF-induced stimulation of DNA synthesis. Our earlier attempts to test this were foiled by the fact that the membranes without the addition of EGF were active in stimulating

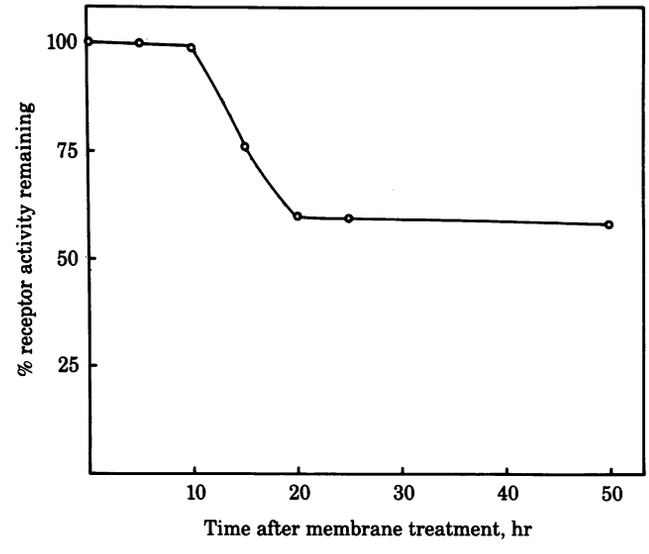

FIG. 4. Stability of the inserted EGF receptor. Monolayers of NR-6 cells in 16-mm dishes were incubated at 26°C for 6 hr with 160 μg of mouse liver membranes (UV sterilized) in 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum. At the end of incubation, the cells were washed free of unbound membranes and incubated at 37°C with 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum for the indicated time periods. Then the monolayers were tested for EGF-receptor activity. One hundred percent receptor activity at 0 time represents binding of 50 fmol of ^{125}I-EGF (31,000 cpm) at 10 nM ^{125}I-EGF. The data depicted here represent the average of two separate experiments.

[^{3}H]thymidine incorporation into DNA (15). However, the peak of DNA synthetic activity induced by the membrane-associated mitogen was over at 30 hr after the removal of liver membranes, and a second peak was not observed at a later point. Because at 30 hr after membrane removal, the NR-6 cells still contained about 60% of the originally inserted EGF receptors (see Fig. 4), the situation permitted a test of EGF receptivity. EGF was added to the cell monolayers at 30 hr after a 6-hr membrane treatment. The cells were then tested for stimulation of [^{3}H]thymidine incorporation into DNA. EGF was found to stimulate DNA synthesis in these membrane-treated NR-6 cells in a concentration-dependent manner (Fig. 5). The degree of stimulation was the same irrespective of whether DNA synthetic rate (1 hr [^{3}H]thymidine pulse) or total DNA synthesis (12 hr [^{3}H]thymidine exposure) was measured. In contrast, NR-6 cells not treated with membranes were totally unresponsive to EGF. The dramatic difference in DNA replicative stimulation that was observed with EGF between membrane-treated and untreated cells was not observed with other growth factors such as fibroblast growth factor or insulin (data not shown).

In order to test whether receptor insertion resulted in a mitogenic responsiveness to EGF, we studied the effect of EGF upon NR-6 cell division (Fig. 6). Membranes alone were slightly stimulatory, producing a 30% increase in cell numbers (15). Addition of 3 nM EGF to NR-6 cells containing inserted receptors resulted in an almost 2-fold increase in cell numbers.

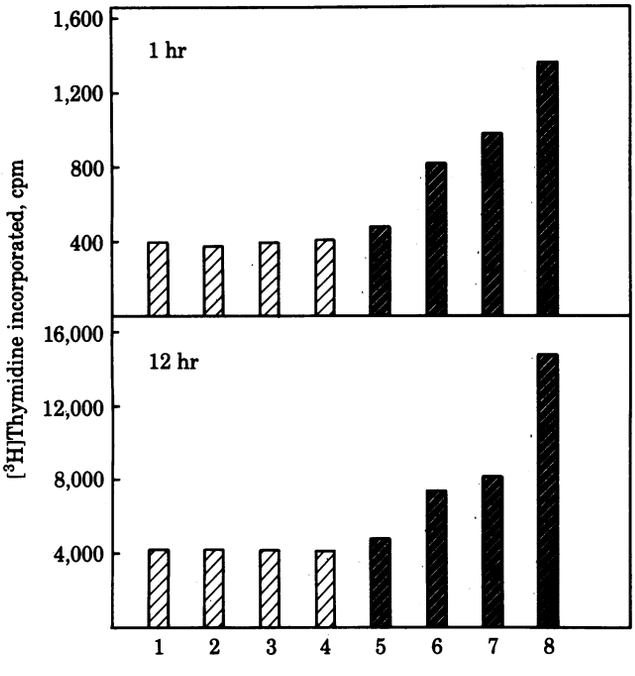

FIG. 5. Inserted receptor and EGF-induced stimulation of DNA replication. Stationary density-inhibited monolayers of NR-6 cells in 16-mm dishes were incubated at 26°C for 6 hr with 160 μg of mouse liver membranes in 0.3 ml of Dulbecco's modified Eagle's medium containing 2% fetal calf serum. At the end of incubation, the cells were washed free of unbound membranes and incubated at 37°C in 0.3 ml of the same medium. After 30 hr of incubation, the medium was removed and the cells were incubated with conditioned Dulbecco's medium containing 2% fetal calf serum and EGF at the indicated concentrations. For the experiments depicted in the lower half of the figure, [^{3}H]thymidine (1 μCi/ml; 0.65 μM) was added at 12 hr after EGF addition, and the incubations were continued for an additional 12 hr. For the experiments depicted in the upper half of the figure, [^{3}H]thymidine was added at 23 hr after EGF addition, and the incubations were continued for additional 1 hr. Bars: 1–4, no membrane treatment; 5–8, membrane treatment; 1 and 5, no EGF treatment; 2 and 6, EGF at 0.3 nM; 3 and 7, EGF at 1 nM; 4 and 8, EGF at 3 nM.

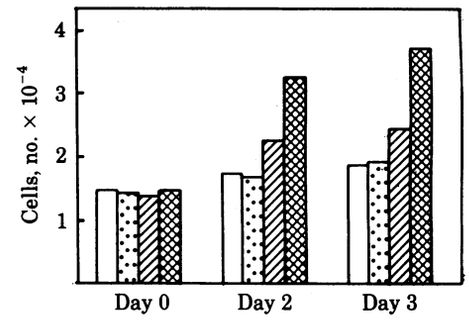

FIG. 6. Inserted receptor and EGF-induced stimulation of cell division. NR-6 cells (in Dulbecco's modified Eagle's medium containing 10% fetal calf serum) were plated into 16-mm dishes at a density of 5 × 10^3 cells per cm^2. After 20 hr, the medium was replaced with Dulbecco's medium containing 0.5% fetal calf serum, and the cells were incubated at 37°C in this low-serum medium for 48 hr. These cells were then incubated at 26°C for 6 hr with 140 μg of mouse liver membranes in 0.3 ml of Dulbecco's medium/0.5% fetal calf serum. At the end of incubation, the cells were washed free of unbound membranes and incubated at 37°C for 30 hr in the same medium. Then, 3 nM EGF was added and incubations were continued at 37°C. Cells were counted on days 0, 2, and 3 after EGF addition; cells were harvested with 0.05% trypsin/0.02% EDTA and counted in a hemocytometer. Cell numbers represent the average of eight hemocytometer counts of cells from duplicate dishes. □, no membrane and no EGF; ⊡, no membrane and 3 nM EGF; ▨, 140 μg of membrane and no EGF; ⊠, 140 μg of membrane and 3 nM EGF.

In contrast, NR-6 cells not treated with membranes were totally unresponsive to EGF. Thus, the inserted EGF receptor behaved like a typical natural mitogen receptor.

Inserted Receptor and Early Biological Responses to EGF. In responsive cells containing EGF receptors, EGF is known to activate phosphofructokinase, a regulatory enzyme in the glycolytic pathway (16). This enzyme in membrane-untreated, control NR-6 cells was not activated by EGF. However, insertion of EGF receptors into these NR-6 cells resulted in a marked responsiveness to EGF as indicated by a 2-fold activation of phosphofructokinase (data not shown).

In responsive cells, EGF is also known to stimulate the uptake of α-aminoisobutyrate. A 20% stimulation of α-aminoisobutyrate uptake was observed when cells containing inserted receptor were incubated with EGF (data not shown). Although low, this stimulation is comparable to that obtained with normal receptor-containing responsive cells (6).

DISCUSSION

The present work shows that a peptide hormone receptor from purified tissue membranes can be transferred to the surface of another cell in the absence of added fusogenic agent. In order to demonstrate that the biological response that is specified by the hepatic EGF receptor is transferred to the recipient NR-6 cell intact, we analyzed for various functions and found that the inserted receptor meets the following criteria: (*a*) the observed receptor gain by NR-6 cells is not due to endogenous protein synthesis, because cycloheximide (1 μg/ml) has no inhibitory effect on this process; (*b*) the inserted receptor is the hepatic receptor as shown by specific covalent radiolabeling studies (Fig. 3); (*c*) the inserted receptor binds to ^{125}I-EGF with high specificity and affinity (Fig. 2); (*d*) it confers upon the hitherto unresponsive NR-6 cells a specific mitogenic responsiveness to EGF as measured by stimulation of DNA replication (Fig. 5) and stimulation of cell division (Fig. 6); (*e*) it is capable of inducing at least two early biological responses to EGF—namely, stimulation of amino acid transport and activation of phosphofructokinase.

Thus, it appears that the transferred EGF receptor acts as a typical natural receptor in many respects, and the membrane-treated variant NR-6 cells function very much like the parent 3T3 cells with respect to responsiveness to EGF. This suggests the existence of a natural physiological mechanism for specific protein transfer from one cell to another. How universal this may be for other cells and other types of receptors remains to be tested. At this point it is difficult to judge whether, in addition to EGF receptor, other molecular components required for message transmission in EGF action are being supplied by the donor hepatic membranes or whether the NR-6 cells also play an active role in supplying some of these components.

Our preliminary time- and temperature-dependence studies (Table 1 and Fig. 1) indicate that preferential insertion rather than nonspecific adhesion is responsible for the receptor gain by the NR-6 cells. Involvement of phospholipids and cytoskeletal structures is suggested by our preliminary observations on inhibition of insertion by pretreatments with low concentrations of phospholipase C or cytochalasin B (unpublished data).

The inserted receptor displayed an exceptional stability profile which greatly facilitated our studies on its biological properties, especially studies on DNA synthesis, cell division, and phosphofructokinase activation. The decay profile (Fig. 4) showed that a large fraction (60%) of the inserted receptor activity was completely resistant to damage up to at least 50 hr of incubation at 37°C. The rest (40%) of the activity was completely stable up to 10 hr but decayed between 10 and 20 hr at 37°C. Regarding this fractional decay, at least two different explanations can be considered: (*a*) existence of two types of EGF receptors in the donor hepatic membranes, one type being more susceptible to dissociation or damage than the other after insertion; (*b*) existence of only a single species of receptor but with two different mechanisms of insertion, leading to differences in insertion stability. Differentiation between these alternatives may become possible in the future when the insertion mechanism is more clearly understood.

The studies described here suggest the existence of a natural (affinity-mediated) mechanism for specific receptor transfer into a receptorless variant cell. Because EGF receptor is an integral membrane protein (detergents are required for its solubilization), it is not easy to visualize a mechanism for its successful insertion in the absence of added fusogens. It is possible that the preferential insertion of EGF receptors over other hepatic proteins is due to a specific NR-6 membrane protein that has a high affinity for the receptor. Such a protein with a tendency to associate with the receptor may also be involved in the biological message transmission mechanism. Therefore, it is of interest to examine the existence of such a protein by using purified EGF receptor as a probe.

We thank Nadia Weschler for assistance in certain experiments. This work was supported by research grants awarded to M.D. from the National Institutes of Health (AM-25819 and AM-25724). M.D. is a Research Career Development Awardee of the National Institutes of Health (AM-00693).

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