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Functional receptors for epidermal growth factor in an epithelial-cell line derived from the rat small intestine

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Epidermal growth factor (EGF) regulates the proliferation of cells of a rat intestinal epithelial-cell line (RIE-1) in culture. Confluent RIE-1 cells were stimulated to proliferate by EGF with a half-maximal effect at 1–2 ng/ml. In contrast, the growth of sparse RIE-1 cells was inhibited by the growth factor. Binding studies at 4°C with ^{125}I-EGF identified two classes of binding sites for EGF on RIE-1 cells, one of high affinity ($K_D = 1.8 \times 10^{-10}$ M; 1.8×10^4 receptors/cell) and one of lower affinity ($K_D = 5.2 \times 10^{-9}$ M; 6.3×10^4 receptors/cell). After binding to the cells at 37°C, ^{125}I-EGF was rapidly internalized and subsequently degraded. Degradation products were released into the medium after a lag of 15–30 min. The degradation of ^{125}I-EGF did not occur at 4°C and was inhibited at 37°C by chloroquine, methylamine or NH_4Cl, but not by colchicine. Exposure of RIE-1 cells to EGF caused a time- and dose-dependent loss of EGF receptors from the cell surface. The recovery of receptors after the removal of EGF was retarded in the absence of serum and prevented by the presence of cycloheximide or actinomycin D. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis separation of the ^{125}I-EGF–receptor complex from RIE-1 cells after covalently cross-linking with disuccinimidyl suberate indicated a receptor of $M_r \simeq 160000$. The demonstration of functional EGF receptors in this cell line provides further evidence that EGF may regulate intestinal-epithelial-cell physiology.

EGF is a small ($M_r \simeq 6000$) polypeptide that is highly mitogenic for various cell types both in culture and *in vivo* (see Carpenter, 1981). The physiological role of EGF remains to be elucidated, but evidence is accumulating that the factor plays a part in regulating cell proliferation and function in the epithelial lining of the gastrointestinal tract. EGF stimulates cell growth in the gastrointestinal tract of mice (Feldman *et al.*, 1978; Scheving *et al.*, 1979, 1980; Chabot & Hugon, 1980; Al-Nafussi & Wright, 1982) and rats (Johnson & Guthrie, 1980; Dembinski *et al.*, 1982; Al-Nafussi & Wright, 1982), and has been shown to influence intestinal-mucosal development and maturation (Chabot & Hugon, 1980; Beaulieu & Calvert, 1981; Malo & Menard, 1982; Oka *et al.*, 1983). Both mouse EGF and human EGF (urogastrone) are potent inhibitors of gastric acid secretion (Bower *et al.*, 1975; Elder *et al.*, 1975).

We have recently isolated an epithelial cell line (RIE-1) from the rat small intestine. RIE-1 cells are untransformed and non-tumorigenic. The epithelial origin of the cells is indicated by their expression of cytokeratin filaments and their binding of monoclonal antibodies directed against epithelial antigens (Blay & Brown, 1984*a*). Here we show that EGF regulates the growth of RIE-1 cells in culture and characterize the interactions of the growth factor with specific cellular receptors. Our findings provide further evidence for a role of EGF in the control of intestinal-epithelial-cell turnover.

A report of some of these results has appeared in abstract form (Blay & Brown, 1983).

Abbreviations used: DSS, disuccinimidyl suberate; EGF, epidermal growth factor; FGF, fibroblast growth factor; FSH, follicle-stimulating hormone (follitropin); GRP, gastrin-releasing peptide; MSA, multiplication-stimulating activity; phosphate-buffered saline, 140 mM-NaCl/2.7 mM-KCl/8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4, pH 7.2; PDGF, partially purified human platelet-derived growth factor; SDS, sodium dodecyl sulphate; Bes, 2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid; DMEM, Dulbecco's modified Eagle's medium.

Experimental

Materials

Culture medium, antibiotics, newborn-calf serum and trypsin were from Flow Laboratories. Nunc tissue-culture dishes were obtained from Gibco. [*methyl*-^{3}H]Thymidine and Na^{125}I were from Amersham International. EGF was prepared from male mouse submaxillary glands by the method of Savage & Cohen (1972). DSS was purchased from Pierce Chemical Co. Actinomycin D, cycloheximide, colchicine, chloroquine and methylamine were from Sigma.

Cells

The derivation and characterization of the RIE-1 cell line have been described (Blay & Brown, 1984*a*). The cells used in these experiments were from cultures of less than passage 20, and were diploid. Stock cultures in 90mm-diameter dishes were maintained in DMEM containing penicillin (100 units/ml), streptomycin (100 μg/ml) and 5% (v/v) newborn-calf serum, and were passaged every 7–10 days at a 1:4 subculture ratio. Cultures for experimental use were seeded in 35mm-diameter dishes at 5×10^4 cells/dish (Blay & Brown, 1984*a*). Cell numbers were determined by using an electronic cell counter (Coulter Electronics).

Binding studies

EGF was iodinated by the lactoperoxidase procedure of Thorell & Johansson (1971). ^{125}I-EGF was separated from the reaction mixture by chromatography on a Sephadex G-25 column, equilibrated and eluted with phosphate-buffered saline, and stored with 0.1% (w/v) bovine serum albumin at −20°C. The specific radioactivity of the preparation was typically 70 μCi/μg.

In the standard binding assay, confluent cultures were rinsed with 1ml of binding medium [DMEM containing 50mM-Bes, 0.1% (w/v) bovine serum albumin and 0.1 μM-KI] and then incubated with fresh binding medium containing ^{125}I-EGF at the concentrations indicated in the Figure legends. Unbound radioactivity was removed by washing the cells (four times) with cold (4°C) phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin and 0.1 μM-KI. The washed cells were solubilized in 1ml of 0.5M-NaOH (60min at 37°C) and total cell-associated radioactivity was determined by γ-radiation counting. Non-specific binding (defined as that which was not displaceable by a 200-fold or greater excess of native EGF) was always less than 3%.

To determine the amounts of intact and degraded ^{125}I-EGF in the binding medium, trichloroacetic acid (10%, w/v, final concn.) was added. After 30min at 4°C the samples were centrifuged (30min, 4°C, 450*g*) and the radioactivity in the precipitate and supernatant was measured. Surface-bound ^{125}I-EGF was determined by acid elution as described by Haigler *et al.* (1980).

Autoradiography

[^{3}H]Thymidine-labelled cultures were fixed and exposed with Kodak AR10 stripping film for 7–10 days at 4°C (Brown & Holley, 1979).

Results

Effect of EGF on the proliferation of RIE-1 cells

Exposure of confluent cultures of RIE-1 cells to EGF in the absence of serum produced a dose-dependent stimulation of DNA synthesis and cell proliferation (Fig. 1). A half-maximal stimulation was elicited at an EGF concentration of 1–2ng/ml of medium, with a maximal effect at approx. 10ng/ml. These EGF concentrations are similar to those required for the stimulation of the proliferation of other cell types in culture (Carpenter, 1981). EGF also stimulated confluent RIE-1 cells in the presence of 5% (v/v) calf serum (results not shown).

RIE-1 cells seeded into medium containing 5% calf serum showed a lag period of variable duration (1–3 days) before beginning to divide. After this lag period the cells proliferated with a population doubling time of ~40h (Fig. 2). In contrast with cells in confluent cultures, RIE-1 cells at low density were not stimulated to proliferate by EGF. Indeed, EGF added at the time of seeding produced an inhibition of cell proliferation and a consequent extension of the lag period (Fig. 2). It is interesting that, in these sparse cultures in which EGF inhibits cell proliferation, the factor induced a pronounced change in cellular morphology. The cells become rounded and elongated, with an appearance suggesting increased cell movement (results not shown). The inhibitory effect of EGF on cell growth was not sustained despite continuous exposure to the factor; after the initial inhibition, the proliferation of EGF-treated cells increased to a maximal growth rate at least as great as that seen during the most rapid growth phase of the untreated cells (Fig. 2). An initial EGF-mediated inhibition of proliferation was also evident in sparse cultures of RIE-1 cells growing in medium containing a lower concentration (0.2%) of serum (Fig. 2). The dual effect of EGF on the growth of RIE-1 cells was related to their density rather than to the time that had elapsed after seeding. In a separate experiment (results not shown), cells seeded at high density showed an immediate increase in number in response to the added growth factor, whereas the growth of cells seeded at low density was again inhibited by EGF.

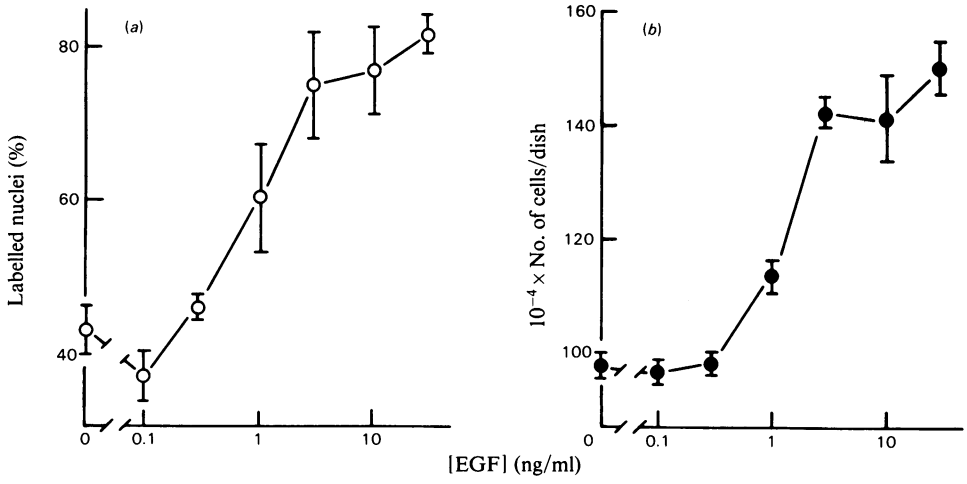

Fig. 1. *Stimulation of DNA synthesis (a) and cell division (b) in confluent RIE-1 cells by EGF*
Cells were grown to confluence in DMEM containing 5% calf serum. The cultures were rinsed with serum-free DMEM and 2ml of serum-free medium containing EGF at the indicated concentration was added per dish. (*a*) Cultures were given [^{3}H]thymidine (final concn. 1 μCi/ml) and processed for autoradiography after a 40h incubation at 37°C. (*b*) Cultures were incubated for 70h at 37°C and the cells counted. The starting density was 97.6 ($\pm$2.6) × 10^4 cells/dish. Points represent the means ($\pm$S.E.M.) of triplicate determinations.

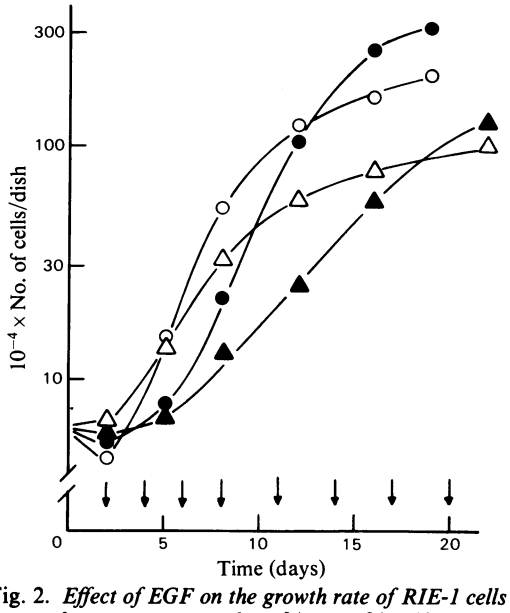

Fig. 2. *Effect of EGF on the growth rate of RIE-1 cells in medium containing either 5% or 0.2% calf serum*
Cells were seeded (5×10^4 cells/dish) in DMEM containing 5% calf serum and incubated overnight to allow cell attachment. The cultures were then rinsed with serum-free DMEM and given 2ml of medium supplemented with 5% (○, ●) or 0.2% (△, ▲), calf serum with (●, ▲) or without (○, △) EGF at a final concentration of 20ng/ml. Fresh medium with or without EGF was added at 2–3-day intervals (arrows). Each point represents the mean cell number from duplicate dishes, presented on a logarithmic scale.

Binding, internalization and degradation of ^{125}I-EGF by RIE-1 cells

The binding of ^{125}I-EGF to RIE-1 cells at 37°C reached a maximum after 30–40min (Fig. 3*a*). Thereafter, there was a steady decrease in the cell-associated radioactivity in spite of the continued presence of ^{125}I-EGF in the medium. This decrease has been observed in other cell types (Carpenter & Cohen, 1976; Aharonov *et al.*, 1978) and has been explained as the consequence of the intracellular degradation of ^{125}I-EGF and release of [^{125}I]iodotyrosine from the cells. The cells have a limited ability to bind fresh ^{125}I-EGF because of receptor translocation from the cell surface (down-regulation). Our finding that trichloroacetic acid-soluble degradation products of ^{125}I-EGF were released from the RIE-1 cells after a short lag period (Fig. 3*a*) is consistent with this proposal. The mean degradation rate of ^{125}I-EGF in this experiment was 250pg/h per 10^6 cells or 2.5×10^4 molecules/h per cell.

In contrast with the results at 37°C, there was no measurable degradation of ^{125}I-EGF by RIE-1 cells at 4°C (Fig. 3*b*). The cell-associated radioactivity increased steadily to reach a maximum after 6h of incubation in the continuous presence of ^{125}I-EGF.

The cellular processing of ^{125}I-EGF by RIE-1 cells at 37°C was examined in more detail. Cells were loaded with ^{125}I-EGF at 4°C, washed free of unbound material, and then transferred to 37°C to allow processing to occur (Fig. 4). Cell-associated

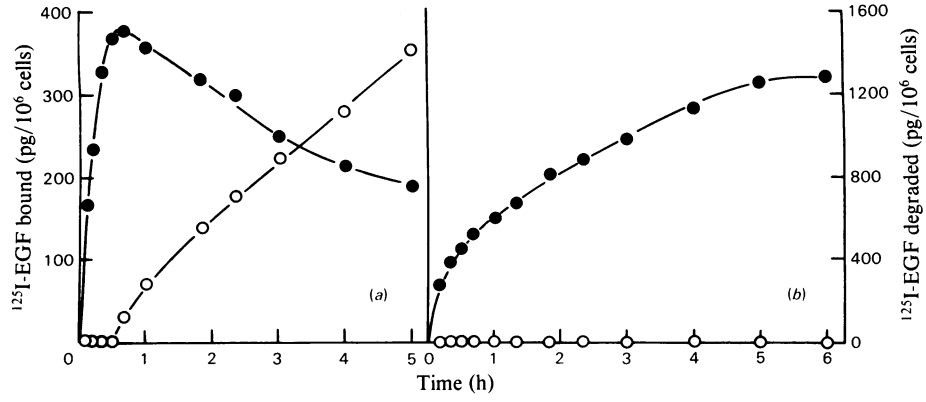

Fig. 3. *Time course of ^{125}I-EGF binding and degradation by RIE-1 cells*
Confluent cultures were incubated at 37°C (*a*) or 4°C (*b*) with 3ml of binding medium containing ^{125}I-EGF (5ng/ml). At the indicated times the medium was removed from the dishes and the amount of trichloroacetic acid-soluble radioactivity in this medium was determined and expressed as ^{125}I-EGF degraded (○). Cell-associated ^{125}I-EGF (●) was measured as described in the Experimental section. Each point represents the mean of triplicate determinations.

Table 1. *Effect of inhibitors of lysosomal function and colchicine on the degradation of ^{125}I-EGF by RIE-1 cells*
Cells were rinsed with binding medium and given 1 ml of binding medium containing the agent at the indicated concentration. After preincubation for 30min at 37°C, ^{125}I-EGF was added (final concn. 5ng/ml) and the cells were incubated for 40min at 37°C. The amount of bound ^{125}I-EGF was measured in several representative dishes (values are means ± S.E.M. of triplicate determinations). The remaining dishes were rinsed with binding medium and incubated with 1 ml of binding medium (containing inhibitor) for a further 3h at 37°C. At the end of this period, the amounts of trichloroacetic acid-insoluble and -soluble radiolabelled material in the medium (intact and degraded ^{125}I-EGF released by the cells respectively) were measured. The radioactivity remaining associated with the cells was also determined. Results are expressed as the proportion of the total radioactivity in the three fractions (means ± S.E.M.; $n = 3$).

Inhibitor	^{125}I-EGF bound (pg/10^6 cells)	Distribution of ^{125}I-labelled material after 3h at 37°C (% of total)		
		Remaining cell-associated	Acid-insoluble in medium	Acid-soluble in medium
None	126.7 ± 8.3	13.0 ± 0.1	9.2 ± 0.2	77.8 ± 0.1
Colchicine (2 μM)	128.5 ± 5.1	14.8 ± 0.3	9.1 ± 0.4	76.1 ± 0.7
Chloroquine (0.2 mM)	162.6 ± 10.0	72.9 ± 0.5	14.7 ± 0.4	12.5 ± 0.5
NH_4Cl (10 mM)	145.5 ± 9.0	58.1 ± 0.3	7.3 ± 0.2	34.6 ± 0.6
Methylamine (10 mM)	160.1 ± 3.0	37.0 ± 0.9	6.9 ± 0.3	56.2 ± 0.9

^{125}I-EGF was initially located predominantly at the cell surface; 83% could be released by cold acetate buffer at pH 2.5. On warming to 37°C there was a rapid ($t_{\frac{1}{2}} \simeq 2.5$min) loss of material from the cell surface. This was partly due to dissociation of intact (trichloroacetic acid-precipitable) ^{125}I-EGF into the medium, but a larger proportion of the ^{125}I-EGF lost from the surface had been taken into the cell and was inaccessible to acid stripping. Intracellular ^{125}I-EGF reached a maximum 15min after the shift to 37°C, after which there was a steady fall accompanied by an accumulation of trichloroacetic acid-soluble degradation products in the medium. A late decline was seen in the amount of intact ^{125}I-EGF that had previously dissociated from the cells into the medium. This presumably reflected partial rebinding of dissociated ^{125}I-EGF to fresh receptors at the cell surface after internalization of the initial receptor–ligand complexes.

Effect of inhibitors of lysosomal function on ^{125}I-EGF degradation by RIE-1 cells

The degradation of ^{125}I-EGF by RIE-1 cells was decreased by agents that interfere with lysosomal function (Table 1). After an initial incubation to allow the binding of ^{125}I-EGF, RIE-1 cells were washed and further incubated for 3h at 37°C before assessing the final distribution of ^{125}I-

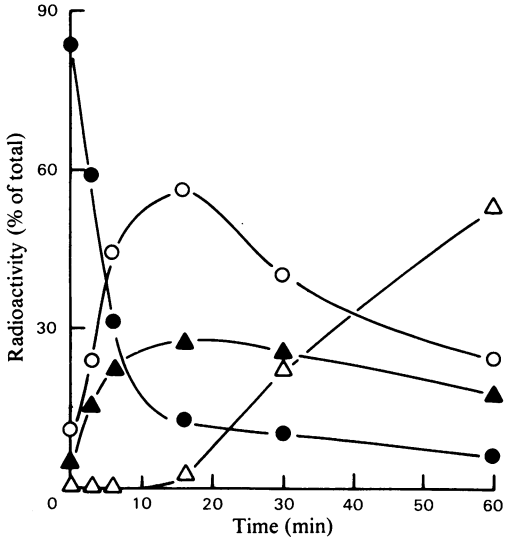

Fig. 4. *Time course of the metabolism of ^{125}I-EGF bound by RIE-1 cells*

Confluent cultures were incubated (60min at 4°C) with 1ml of binding medium containing 5ng of ^{125}I-EGF/ml. The cells were washed three times with cold phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin and 0.1 μM-KI, given 1ml of binding medium, and incubated at 37°C. At the indicated times the medium was collected and the cells were rinsed with 330 μl of binding medium. This rinse was pooled with the first medium collection and the trichloroacetic acid-insoluble (▲) and trichloroacetic acid-soluble (△) radioactivity in the pools were determined; the former represents ^{125}I-EGF dissociating intact from the cell surface and the latter represents the release of degraded ^{125}I-EGF (Brown *et al.*, 1979*b*). The cells were treated with 700 μl of 0.2M-acetate buffer, pH2.5, containing 0.5M-NaCl for 6min at 4°C and then washed with a further 400 μl of the same solution. The acid washes were combined and counted for radioactivity to determine the amount of surface (i.e. acid-releasable) ^{125}I-EGF (Haigler *et al.*, 1980) (●). The remaining cell-associated radioactivity (○) was recovered by solubilizing the cells in 1ml of 1M-NaOH for 60min at 37°C. Each point represents the mean value ($n = 3$) of ^{125}I-EGF in each of the four fractions described above, expressed as a percentage of the total radioactivity.

labelled material. In control cultures, nearly 80% of the radioactivity recovered at the end of this period was as degradation products in the medium. Inhibitors of lysosomal function decreased ^{125}I-EGF degradation with a consequent increase in the remaining cell-associated radioactivity. These results suggest that RIE-1 cells degrade ^{125}I-EGF in the lysosomes. Chloroquine (0.2mM) was a more effective inhibitor of ^{125}I-EGF degradation than NH_4Cl (10mM), which in turn was more effective than methylamine (10mM). In the presence of chloroquine the amount of degraded material at the end of the 3h incubation was 16% of that in the absence of the inhibitor.

The presence of these inhibitors increased the amount of ^{125}I-EGF binding to RIE-1 cells in the initial incubation (Table 1). Since these agents do not affect ^{125}I-EGF binding to membrane-enriched fractions of RIE-1 cells (J. Blay & K. D. Brown, unpublished work), this is probably an indirect effect of blocking the normal processing pathway. ^{125}I-EGF accumulates within the cells rather than being degraded.

Colchicine has been shown to inhibit the degradation of ^{125}I-EGF by 3T3 fibroblasts (Brown *et al.*, 1980), possibly by disrupting a microtubule-dependent delivery of ^{125}I-EGF to the lysosomes. Colchicine, at a concentration which is maximally effective in inhibiting ^{125}I-EGF degradation by 3T3 cells, failed to affect ^{125}I-EGF degradation by RIE-1 cells (Table 1). This concentration of the drug was effective in blocking RIE-1 cell mitosis (result not shown). The delivery of ^{125}I-EGF to lysosomes in RIE-1 cells may therefore be less dependent on microtubule function than in 3T3 cells.

Characteristics of binding sites for EGF on RIE-1 cells

The binding of ^{125}I-EGF to RIE-1 cells was decreased by the presence of unlabelled EGF; a 10-fold excess produced 85% inhibition (Table 2). ^{125}I-EGF binding was not decreased by a number of other growth factors and hormones added in large excess (Table 2). A small, but statistically significant, decrease in ^{125}I-EGF binding to RIE-1 cells was seen with vasopressin at 1 μg/ml ($P < 0.05$). Since vasopressin did not inhibit the binding of ^{125}I-EGF to isolated RIE-1 cell membranes (J. Blay & K. D. Brown, unpublished work), the inhibition of binding is not likely to be a direct action, but rather an indirect effect on EGF-receptor expression by the intact cell.

The binding of ^{125}I-EGF to RIE-1 cells (measured at 4°C to prevent cellular processing) was saturable (Fig. 5) and reversible (results not shown). We have previously reported (Blay & Brown, 1983) that RIE-1 cells have a single class of EGF receptors. However, a Scatchard (1949) plot of data obtained over the concentration range 0.01–100ng/ml showed a non-linear relationship (Fig. 5, inset), which could be fitted to a model of two classes of binding site with different affinities. The calculated apparent dissociation constants (K_D) and maximal binding capacities ($B_{max.}$) for the two classes were: high affinity, $K_D = 1.8 \times 10^{-10}$M, $B_{max.} = 180$pg/10^6 cells; and low

Table 2. *Effect of growth factors and hormones on ^{125}I-EGF binding to RIE-1 cells at 37°C*

Confluent cultures of RIE-1 cells were rinsed with binding medium and incubated for 40 min at 37°C with 1 ml of binding medium containing 5 ng of ^{125}I-EGF/ml and the additions shown. The cell-associated ^{125}I-EGF was then measured as described in the Experimental section. The values are means (±S.E.M.) of quadruplicate determinations.

Addition	Concn.	^{125}I-EGF bound (pg/dish)
None	–	281.7±11.9
EGF	50 ng/ml	43.6±1.4
PDGF	16 μg/ml	266.6±4.4
FGF	100 ng/ml	274.2±12.4
Insulin	10 μg/ml	240.2±10.7
MSA	1 μg/ml	244.6±2.3
FSH	1 μg/ml	253.8±13.8
Oxytocin	1 μg/ml	267.9±10.2
Vasopressin	1 μg/ml	232.9±2.3
Bombesin	0.16 μg/ml	265.9±15.5
GRP	0.31 μg/ml	253.7±9.4
Dexamethasone	1 μg/ml	259.6±9.7
Retinoic acid	0.3 μg/ml	258.0±13.4

affinity, $K_D = 5.2 \times 10^{-9}$ M, $B_{max.} = 630$ pg/10^6 cells. The total binding capacity (810 pg/10^6 cells) gives a value of $\sim 8 \times 10^4$ receptors per cell, assuming a 1:1 molar stoichiometry of binding of ^{125}I-EGF to receptor.

Regulation of EGF-binding capacity of RIE-1 cells

Exposure of the RIE-1 cells to EGF resulted in a decreased capacity to bind ^{125}I-EGF subsequently (Fig. 6). Scatchard analysis of binding data confirmed that this was due to a decrease in $B_{max.}$ with no change in the binding affinities (results not shown). The time course of receptor down-regulation is shown in Fig. 6(*a*). The cellular binding capacity was decreased by 50% after a 3 h exposure of the RIE-1 cells to EGF at 10 ng/ml. This down-regulation of EGF receptors was dose-dependent (Fig. 6*b*), with a half-maximal effect at an EGF concentration of approx. 1 ng/ml, which is close to the concentration required for half-maximal stimulation of the growth of RIE-1 cells.

The recovery of cellular receptors after EGF-induced receptor down-regulation was investigated (Fig. 7). An 80% decrease in the cellular binding capacity was induced by treatment with EGF. When the cells were incubated in medium containing 5% calf serum, the recovery of EGF receptors was complete 8 h after the removal of EGF. By comparison, the recovery of EGF receptors by RIE-1 cells incubated in serum-free medium (containing 0.1% bovine serum albumin) was slow. Both actinomycin D and cycloheximide

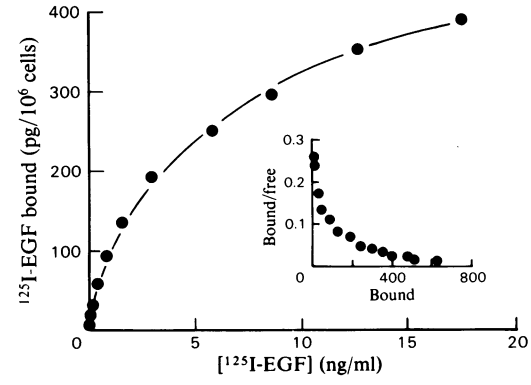

Fig. 5. *Binding of ^{125}I-EGF to RIE-1 cells at 4°C as a function of ^{125}I-EGF concentration*

Confluent monolayers of RIE-1 cells were rinsed with binding medium and incubated with 1 ml of binding medium containing increasing concentrations of ^{125}I-EGF, for 5 h at 4°C. The equilibrium concentration of free ^{125}I-EGF was calculated from the radioactivity measured in a sample of the medium taken at the end of the incubation period. Cell-associated ^{125}I-EGF was measured as described in the Experimental section. The results have been corrected for non-specific binding (not displaced by a minimum 200-fold excess of unlabelled EGF). Each point represents a single value for ^{125}I-EGF binding. The inset shows a Scatchard plot of the data.

blocked the recovery of cellular EGF receptors in medium containing calf serum, indicating that synthesis of both RNA and protein is required for recovery. Whether receptor synthesis itself is required, or whether protein synthesis is simply required for the insertion of recycled receptors, is not known.

Identification of the receptor on RIE-1 cells

The EGF receptor on RIE-1 cells was identified by covalently coupling ^{125}I-EGF to the binding site(s) with the homobifunctional cross-linking reagent DSS. The covalently stabilized complex(es) were then separated by SDS/polyacrylamide-gel electrophoresis and localized by autoradiography (Fig. 8). The major cross-linked complex migrated under reducing conditions with an apparent $M_r \simeq 165000$ (Fig. 8, lanes B–E). Minor ^{125}I-labelled bands ($M_r \simeq 140000$, 115000 and 90000) were also seen in some experiments. At high concentrations of DSS (1 mM or greater) high-M_r material was also labelled (Fig. 8, lanes D and E), presumably representing aggregates of the ^{125}I-EGF–receptor complex cross-linked to other proteins. The incorporation of ^{125}I-EGF into the complex was blocked by unlabelled EGF (Fig. 8, lane F), but not by various other growth factors and

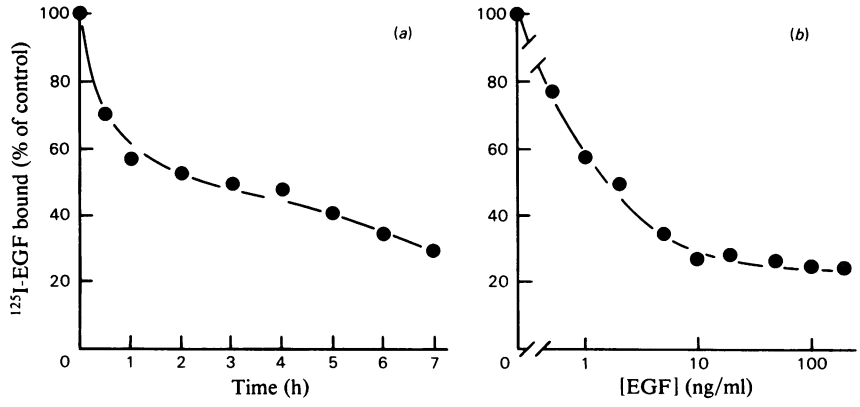

Fig. 6. *Time course (a) and dose–response (b) for the down-regulation of EGF receptors in RIE-1 cells*
(*a*) Unlabelled EGF (10ng/ml) was added directly to the growth medium of confluent cells and the cultures were incubated at 37°C. At the indicated times the cells were washed and incubated with 1ml of binding medium for 60min at 37°C to allow the clearance of cell-surface-bound EGF. ^{125}I-EGF binding was then measured after a 40min incubation with binding medium containing 1ng/ml of ^{125}I-EGF. (*b*) Confluent RIE-1 cells were incubated with the indicated concentration of unlabelled EGF for 7h. The cells were washed and ^{125}I-EGF binding was measured as described above. Points represent the mean values ($n = 3$) obtained for ^{125}I-EGF binding expressed as a percentage of the mean control value for binding to untreated cells.

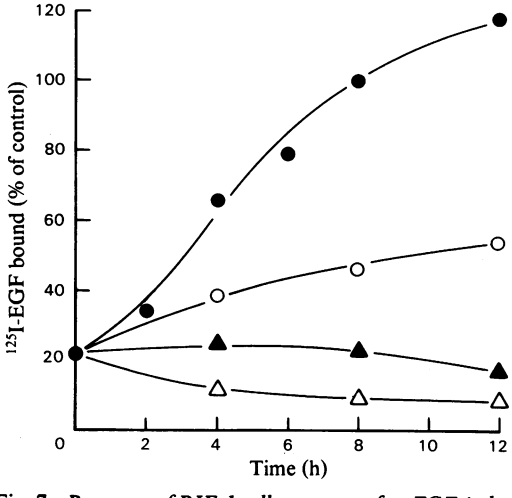

Fig. 7. *Recovery of RIE-1 cell receptors after EGF-induced down-regulation*
Cells in depleted growth medium were incubated with 50ng of unlabelled EGF/ml for 8h at 37°C, washed, and incubated with binding medium for 60min at 37°C to allow the clearance of cell-surface-bound EGF. The cultures were then incubated at 37°C for the indicated times with 2ml of DMEM containing 5% calf serum (●), 0.1% bovine serum albumin (○), 5% calf serum and 1 μg of actinomycin D/ml (▲), or 5% calf serum and 20 μg of cycloheximide/ml (△). ^{125}I-EGF binding was subsequently measured after a 40min incubation with binding medium containing 1ng of ^{125}I-EGF/ml and is expressed as a percentage of the binding to control cultures not pretreated with EGF.

hormones present in large molar excess, including PDGF, FGF, insulin, MSA, vasopressin and oxytocin (results not shown).

Discussion

Studies of intestinal-epithelial-cell growth in the whole animal are difficult because of the complexity of intestinal tissue structure, and the numerous influences that can affect cell turnover in the gastrointestinal tract (see Robinson *et al.*, 1982). The use of cultured cells makes it possible to examine the control of proliferation by individual factors under defined conditions. EGF has been reported to enhance the growth of cultured intestinal epithelial cells (Quaroni & May, 1980), but a detailed examination is lacking. We have therefore investigated the actions of EGF on a normal epithelial-cell line derived from the rat small intestine (Blay & Brown, 1984*a*).

Epithelial cells in the rat small intestine *in vivo* are likely to be exposed to luminal EGF. EGF is present in high concentrations in the salivary glands (Moore, 1978; Gresik *et al.*, 1979), and is secreted in saliva (Byyny *et al.*, 1974; Hirata & Orth, 1979). It is stable to acid (Savage & Cohen, 1972) and remains biologically active after exposure to trypsin (Savage *et al.*, 1972), so would resist degradation in the gut lumen. Indeed, radiolabelled EGF administered orally to suckling rats can be recovered intact from the intestinal lumen (Thornburg *et al.*, 1984). Furthermore, EGF has

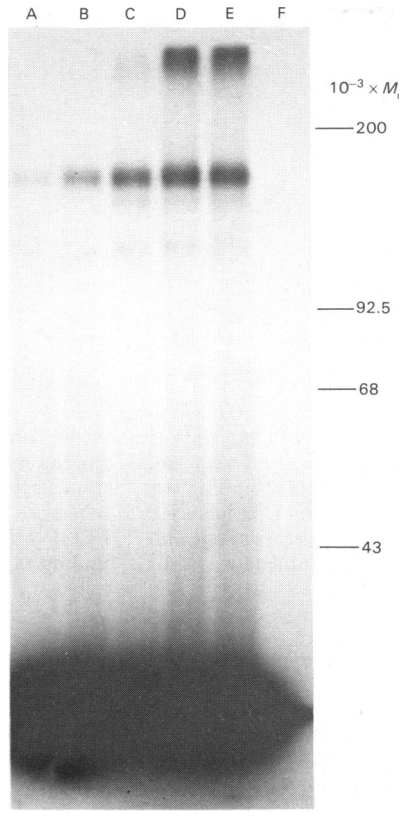

Fig. 8. *Cross-linking of* ^{125}*I-EGF to its receptor on RIE-1 cells*

Cells were incubated with 20ng of ^{125}I-EGF/ml in binding medium for 40min at 37°C, cooled to 4°C and washed twice with cold phosphate-buffered saline containing 0.1% (w/v) bovine serum albumin and 0.1 μM-KI. The cultures were rinsed and given 1ml of cold phosphate-buffered saline containing 0.9mM-Ca^{2+} and 0.5mM-Mg^{2+}. DSS in dimethyl sulphoxide, or dimethyl sulphoxide alone, was added to give the DSS concentrations indicated with a final dimethyl sulphoxide concentration of 3% (v/v). After 15min at 4°C the cross-linking reaction was quenched by replacement of the solution with 1ml of cold 150mM-Tris, pH7.4. The cells were rinsed and scraped into 0.5ml of cold phosphate-buffered saline and centrifuged at 300*g* for 10min at 4°C. The cell pellet was lysed in 60μl of cold lysis buffer (10mM-NaCl/1.5mM-$MgCl_2$/1% Nonidet P40/10mM-Tris, pH7.4) and the nuclei were removed by centrifugation at 700*g* for 10min at 4°C. The supernatant was supplemented with concentrated (5×) electrophoresis sample buffer (Laemmli, 1970), and heated to 100°C for 4min. Portions containing equivalent radioactivities were subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions using a 4% (w/v)-acrylamide stacking gel and 7.5% separating gel. The gel was fixed in 50% (v/v) methanol in 10% (v/v) acetic acid, dried on to filter paper, and exposed to Kodak X-Omat S film at −70°C with an Ilford Fast Tungstate intensifying screen. Lanes A–E, 0, 0.1mM, 0.3mM, 1mM and 3mM-DSS; Lane F, 500-fold excess of native EGF present during ^{125}I-EGF binding, 3mM-DSS. The M_r markers shown are myosin H-chain (M_r 200000), phosphorylase *b* (92500), bovine serum albumin (68000) and ovalbumin (43000).

been identified in Brunner's glands (Elder *et al.*, 1978; Heitz *et al.*, 1978) and is secreted into the duodenal lumen (Kirkegaard *et al.*, 1983). Skov Olsen & Nexø (1983) have reported the concentration of EGF in rat duodenal juice to be ~200pM (~1.2ng/ml), within the range in which mouse EGF stimulates RIE-1 cell proliferation.

EGF stimulates the proliferation of confluent RIE-1 cells in serum-free conditions without the addition of other macromolecular growth factors. This contrasts with the response to EGF of fibroblastic cells, which is only weak in the absence of additional growth-promoting factors (Brown *et al.*, 1979*a*). A further feature of the RIE-1 cell response to EGF is that cells exposed to EGF are able to grow to a higher maximal density than untreated cells (Fig. 2). Such an increase in the saturation density has also been reported for cultured normal mammary epithelial cells treated with EGF (Kirkland *et al.*, 1979). In addition to its effects on cell proliferation, EGF can affect the morphology and movement of RIE-1 cells (see below) and may therefore permit a higher saturation density by influencing cellular packing within the monolayer.

In sparse cultures, EGF slows RIE-1-cell proliferation. This inhibition of growth is accompanied by striking changes in the appearance of the cells which round, elongate, and adopt a morphology suggesting increased migration (results not shown). We have previously established that EGF markedly stimulates and directs the movement of RIE-1 cells in an assay *in vitro* of cell migration (Blay & Brown, 1984*b*). The unexpected observation that EGF retards the growth of RIE-1 cells at low density, in contrast with increasing the proliferation of confluent cells, may indicate that cells are diverted from a mitogenic response when responding to the growth factor by increased locomotion. Such an antagonism between proliferation and movement is also suggested by the demonstration (Grotendorst, 1984) that the migratory response of fibroblasts to PDGF is decreased under conditions of rapid cell growth.

RIE-1 cells bind ^{125}I-EGF with high affinity and specificity at the cell surface. These findings confirm and extend other work which indicates the presence of EGF-binding sites on intestinal epithelial cells. Chabot *et al.* (1982) have used autoradiography of tissue sections to show binding of ^{125}I-EGF in the rat small intestine, associated mainly

with the epithelial cells of villi and crypts. Forgue-Lafitte *et al.* (1980) have investigated ^{125}I-EGF binding to freshly isolated cells released from rat intestine, using EDTA. The number of specific binding sites measured was very low ($\sim 1 \times 10^3$ receptors/cell) compared with the number of EGF receptors on RIE-1 cells ($\sim 8 \times 10^4$/cell). One explanation for this low extent of ^{125}I-EGF binding to freshly isolated cells may be the rapid inactivation of binding sites during their release from the mucosa (see Forgue-Lafitte *et al.*, 1980). In addition, receptor number *in vivo* may be lower because of down-regulation caused by continuous exposure to endogenous EGF.

Initial results (Blay & Brown, 1983) suggested that RIE-1 cells possessed a single class of EGF receptors ($\sim 1 \times 10^5$ sites/cell) with an apparent K_D of $\sim 2 \times 10^{-9}$M. These values are similar to those reported (2×10^4–2×10^5 sites/cell; $K_D = 2 \times 10^{-10}$–7×10^{-9}M) for EGF binding to a wide variety of cells (Carpenter *et al.*, 1975; Vlodavsky *et al.*, 1978; Phillips *et al.*, 1983). More detailed analyses have demonstrated that two classes of EGF receptors, differing in their binding affinities ($K_{D_1} = 1.8 \times 10^{-10}$M, $K_{D_2} = 5.2 \times 10^{-9}$M) are present on RIE-1 cells. Curvilinear Scatchard (1949) plots indicative of more than one receptor class have been presented for ^{125}I-EGF binding to other cell types (Osborne *et al.*, 1982; Richards *et al.*, 1983; Collins *et al.*, 1983). The K_D values of the two receptor classes are similar to those given for RIE-1 cells, and the proportion of high-affinity sites ranges from 20 to 40%.

After binding at the cell surface, ^{125}I-EGF is taken within the RIE-1 cells, after an interval of approx. 15 min, broken down, with the subsequent release of degradation products into the medium. The inhibition of this degradation by inhibitors of lysosomal function shows that it takes place in the lysosomes. Internalization and lysosomal hydrolysis of ^{125}I-EGF have been described for various other cell types (see Carpenter, 1981).

^{125}I-EGF that has bound to RIE-1 cells can be cross-linked *in situ* by using DSS, with the formation of a ~ 165 kDa complex of ^{125}I-EGF with its putative binding component. A small amount of this complex is formed in the absence of cross-linker (Fig. 8, lane A). Spontaneous, low-efficiency, covalent bonding of ^{125}I-EGF to its binding site in the absence of chemical cross-linking agents has been noted by other workers (Baker *et al.*, 1979; Linsley *et al.*, 1979; Comens *et al.*, 1982). Assuming a 1:1 molar stoichiometry of binding, and subtracting the M_r of ^{125}I-EGF from the M_r of the labelled complex, these results suggest a binding component of ~ 160 kDa. ^{125}I-EGF binding to RIE-1 cell membranes is markedly decreased by proteinase pretreatment of the membranes (results not shown). The receptor for EGF on RIE-1 cells therefore seems to be a protein of apparent M_r 160000. This value is similar to those reported for the M_r of the EGF receptor on other cell types (see Carpenter, 1981).

We have shown that RIE-1 cells have specific high-affinity receptors for EGF, and that the proliferation of these cells is regulated by the growth factor. Together with our previous finding that EGF influences the migration of RIE-1 cells (Blay & Brown, 1984*b*), these results provide further evidence for the importance of EGF in intestinal-epithelial-cell physiology.

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