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In "Accumulation of Epidermal Growth Factor within Cells Does Not Depend on Receptor Recycling," by A. Christie King, Robert A. Willis, and Pedro Cuatrecasas, pp. 840-845, Fig. 1 was repeated as Fig. 2. For our readers' convenience, beginning on the following page, the complete corrected article is reproduced.

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ACCUMULATION OF EPIDERMAL GROWTH FACTOR WITHIN CELLS DOES NOT DEPEND ON RECEPTOR RECYCLING

A. Christie King, Robert A. Willis and Pedro Cuatrecasas

Department of Molecular Biology, Wellcome Research Laboratories, Research Triangle Park, N.C. 27709

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Summary. Quiescent cells seemingly have a constant number of surface epidermal growth factor receptors. However, exposure of cells to agents which interfere with normal protein turnover suggests that these receptors are internalized and degraded with an apparent half-life of ~6 hours. We show that the time course of maximal accumulation of ligand-receptor complexes is not altered under conditions where degradation of the ligand is inhibited, indicating that no degradation occurs during its first hour of exposure to cells. We also conclusively demonstrate that epidermal growth factor receptors are not recycled during the initial uptake of the ligand, and that a component of pinocytosis of this growth factor is dependent on de novo protein synthesis.

Introduction. Many physiologically relevant macromolecules are selectively transported into cells by adsorptive pinocytosis (1), including the carrier proteins, transcobalamin II (2) and low density lipoprotein (1,3), the carbohydrate-specific recognition systems (4), lysosomal hydrolases (5,6), and the polypeptide hormone, epidermal growth factor (EGF)[1] (7,8). In each instance cited, pinosomes are targeted for fusion with lysosomes where the ligand is selectively degraded (1-8). A common feature of most of these transport mechanisms is that the receptor is spared from degradation and is recycled back to the plasma membrane (1-6). The single exception found to date is for EGF receptors where extended incubations with [^{125}I]EGF may result in a dramatic (>90%) and rapid ($t_{\frac{1}{2}}$ 30 min) loss of receptors (7-11). Thus, the specific uptake mechanism for EGF ultimately results in loss of surface receptors, and therefore differs significantly from other systems studied. It has not yet been conclusively demonstrated whether the initial accumulation of [^{125}I]EGF by cells depends on receptor recycling. We now present a comparison of the turnover of the EGF receptor in the resting state and after binding of [^{125}I]EGF

[1] Abbreviations: EGF, epidermal growth factor; EDTA, ethylene dinitrilo-tetracetic acid; BSA, bovine serum albumin; MEM, minimal essential medium.

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and demonstrate that the initial accumulation of EGF within cells does not involve receptor recycling, but that a small component of pinocytosis of [^{125}I]EGF depends on de novo protein synthesis.

Materials and Methods. Materials and Cell Culture. A cell line from an oral epidermal carcinoma (KB) was used for all binding assays and was cultivated as described (10). EGF was purified (12) in our laboratory from male mouse submaxillary glands (Pel Freeze) and iodinated with chloramine T (13) to a specific activity of 20-40 Ci/mmol.

^{125}I-EGF binding assay. Cells were harvested from culture flasks with two washes of 0.05% EDTA in phosphate buffered saline, 0.1% BSA, washed twice, and resuspended into Krebs Ringer, 0.1% BSA or Hanks MEM, 2% serum. Cells were preincubated under the conditions described (Figure 1), and at the indicated times, aliquots of cells were removed, incubated with [^{125}I]EGF and filtered through 1.0 μ Millipore filters with 2 consecutive, 10 ml washes. The experiment shown in Figure 2 involved no preincubation period, and the drugs were added simultaneously with the ^{125}I-EGF. Non-specific binding was determined by including 5 μg/ml unlabeled EGF in parallel incubations. 10 mM $MeNH_2$ blocks degradation of cell-associated ^{125}I-EGF by >95% (10) and 10 μg/ml cycloheximide blocks all protein synthesis within ~2 min.

Results and Discussion. Effect of $MeNH_2$ and Cycloheximide on Surface Receptors. To determine the effect of inhibition of the normal turnover of EGF receptors, intact KB cells were preincubated with 10 μg/ml cycloheximide or 10 mM $MeNH_2$ with and without 10 ng/ml unlabeled EGF. At the indicated times, an aliquot of cells was assayed for its capacity to accumulate [^{125}I]EGF at 37° for 30 min (Figure 1). Untreated cells show no change in pinocytic rate throughout the experiment (12 hours). Preincubation of KB cells with cycloheximide or $MeNH_2$ results in a loss of the ability of cells to accumulate [^{125}I]EGF with a half-life ranging from 4-6 hours. The relative rates of loss in cells treated with $MeNH_2$ or cycloheximide are always approximately equivalent, the observed rates are not potentiated by increasing the concentration of the drug, and the rate of loss in the presence of both compounds is enhanced over that observed in the presence of only one (not shown). These results demonstrate that hormone receptors may be internalized even in the absence of ligand binding as a result of normal receptor turnover, with a turnover rate unusually rapid for membrane macromolecules (14).

Preincubation of cells with subsaturating levels of EGF with cycloheximide or $MeNH_2$ potentiates the rate of loss of pinocytic capacity approximately 10-fold ($t_{\frac{1}{2}}$ ~30 min) over the rate observed in the absence of receptor synthesis

or degradation ($t_{\frac{1}{2}}$ ~6 hr). This loss is biphasic, with the rate after 90 minutes returning to that observed with $MeNH_2$ or cycloheximide alone. Thus, the occupation of receptors by EGF only transiently stimulates the rate of internalization of surface receptors. Thereafter, their rate of loss returns to basal levels. After EGF induces internalization of a large proportion of surface receptors, there remains a residual population which is inexplicably resistant to degradation (7-10). It is not presently known what role, if any, these residual receptors play in mediating those processes stimulated by EGF. However, we have recently found that these receptors act to specifically direct EGF into cells for up to 24 hours after the down regulation process is seemingly complete (unpublished results). Thus, the process of internalization of EGF is not complete 2 to 4 hours after its initial exposure to cells, but is a long-term consequence of EGF binding.

Effect of $MeNH_2$ and Cycloheximide on Pinocytosis of [^{125}I]EGF. Inhibition of protein synthesis with 10 μg/ml cycloheximide has no effect on the initial rate of uptake of saturating concentrations of [^{125}I]EGF (Fig. 2). After approximately 20-30 minutes, however, EGF pinocytosis decreases slightly in the presence of cycloheximide (■). These results suggest that after the initial binding of EGF, either new receptors are synthesized and inserted into the plasma membrane or that a small intracellular pool of receptors exists for which insertion requires protein synthesis of a rapidly turning over factor.

On the contrary, incubation of cells with $MeNH_2$ causes no alteration in the time course of initial accumulation of [^{125}I]EGF from that of untreated cells (Fig. 2). At times later than 1 hour, however, when [^{125}I]EGF is usually rapidly degraded (7-10), its accumulation into cells is stimulated with $MeNH_2$ 2- to 3-fold above the maximal level observed in untreated cells (10). The results from the time courses obtained when surface receptors are maximally occupied by EGF (Fig. 2) indicate that even though [^{125}I]EGF is internalized in the presence of $MeNH_2$, it is not degraded, since the maximal values of pinocytosis with and without the amine are identical. These data strongly

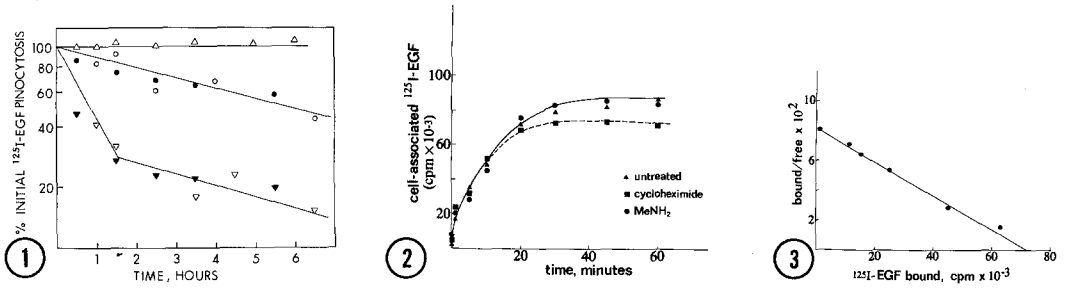

Figure 1. Effect of $MeNH_2$, Cycloheximide and EGF Treatment on EGF Surface Receptor Population. KB cells were incubated in the presence of 10 μg/ml cycloheximide with (▼) or without (●) 10 ng/ml EGF, or 10 mM $MeNH_2$ with (▽) or without (○) 10 ng/mg EGF. At the indicated time, an aliquot of the cell suspension was removed and incubated with 50 ng/ml ^{125}I-EGF for 30 min to determine the capacity for receptor-mediated internalization. 5 μg/ml unlabeled EGF was included in parallel incubations to determine non-specific [^{125}I]EGF association. The pinocytic capacity of untreated cultures is shown (Δ).

Figure 2. Effect of $MeNH_2$ and Cycloheximide on Pinocytosis of EGF. KB cells were incubated with a saturating concentration of [^{125}I]EGF (60 ng/ml) with and without 10 μg/ml cycloheximide or 10 mM $MeNH_2$. At different times of incubation, free [^{125}]EGF was removed from the cultures by filtration and the amount of cell-associated radioactivity was determined as described (10).

Figure 3. Scatchard Plot of [^{125}I]EGF Binding to KB Cells. KB cells (4 x 10^5 cells/ml) were incubated at 4° for 3 hr with varying concentrations of [^{125}I]-EGF (0.05-60 nM). At this time, cells were filtered and the specifically bound radioactivity determined as described in Methods. Equilibrium binding of [^{125}I]EGF is reached after ~2.5 hours at 4°.

suggest that degradation of [^{125}I]EGF requires a lag of about 1 hour from its initial cellular exposure, presumably the time required for translocation to lysosomes. Thereafter, the EGF-receptor complex is rapidly degraded (7-10,15).

Do EGF receptors recycle? The time course of uptake of saturating concentrations of [^{125}I]EGF is only slightly affected by the presence of cycloheximide at times up to 1 hour (Fig. 2). From the rate of loss of surface receptors in the presence of cycloheximide (Fig. 1), an approximate half-time for receptor synthesis and insertion can be estimated to be about 6 hours. Thus, after a 1 hour incubation, the contribution of [^{125}I]EGF associated with newly synthesized receptors is estimated to be about 10% of the total receptor population. The number of surface receptors on KB cells is most reliably estimated by Scatchard analysis (16) obtained under equilibrium conditions (4°, 3 hr). KB cells have a single class of receptors when measured under these conditions having approximately 35,000 receptors with an affinity (Kd) of approximately 0.8 to 1.0 nM (Fig. 3). The difference in uptake of [^{125}I]EGF when cycloheximide

is present (Fig. 2) is just slightly greater than that predicted by the apparent rate of receptor synthesis (Fig. 1). These results confirm that a small component of uptake of EGF may be dependent on de novo receptor synthesis. Since the [^{125}I]EGF which accumulates within cells during the time course measured is not degraded (fig. 2, ref. 10), the accumulation measured under these conditions is not an underestimate of the maximal capacity of the system due to degradation of the radioligand as described for other systems (17). Furthermore, the maximal uptake in the presence of cycloheximide (Fig. 2) exactly correlates with the total receptor number determined by equilibrium binding at 4° (Fig. 3). Therefore, a reliable conclusion is that the initial (1 hour) accumulation of EGF receptors does not depend on recycling when they are maximally occupied.

The results presented in this report demonstrate that (a) the EGF receptor is internalized in the resting state and is continually and rapidly resynthesized and inserted into the plasma membrane at an equivalent rate (Fig. 1). This report also makes the observations that (b) EGF receptors may be continually synthesized and inserted into the plasma membrane even after the initial exposure of cells to EGF (Fig. 2), (c) that the rate of receptor internalization is only transiently stimulated by binding of EGF to its receptor (Fig. 1), and (d) the translocation of EGF to lysosomes may require approximately 1 hour from the initial time of incubation (Fig. 2). Comparison of the uptake data obtained at 37° in the presence of cycloheximide (Fig. 2) with the binding data obtained at 4° (Fig. 3) shows (e) that there are no cryptic or intracellular EGF binding sites under these incubation conditions and (f) that the initial and maximal obtainable accumulation of [^{125}I]EGF into cells does not require recycling of surface receptors.

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