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Epidermal Growth Factor Stimulates Fluid Phase Endocytosis in Human Fibroblasts Through a Signal Generated at the Cell Surface

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We have investigated the stimulation of fluid phase endocytosis by epidermal growth factor (EGF) in normal human fibroblasts using ^{125}I-labeled polyvinylpyrrolidone (^{125}I-PVP) as a fluid phase marker. We found that EGF initially induced a threefold increase in the rate of ^{125}I-PVP uptake. This initial burst of fluid uptake terminated within 10 min. Thereafter, the rate of fluid uptake in EGF-treated cells was approximately 40% higher than in control cells. To identify the cellular site of EGF action in stimulating fluid phase endocytosis, we examined the kinetics of the induction of this response as well as the kinetics of cell surface binding and internalization of ^{125}I-EGF. Although there was no detectable lag between binding of EGF to the cell surface and its internalization, the kinetics of the two processes were quite different. Significantly, the kinetics of induction of ^{125}I-PVP uptake matched the kinetics of binding of ^{125}I-EGF to its cell surface receptors, indicating that the signal for the increase in fluid phase endocytosis is generated at the cell surface. To determine if EGF-stimulated fluid phase endocytosis was related to EGF-stimulated endocytosis of its own receptor, we compared the EGF dose dependency and time course of the two processes. Although the stimulated endocytosis of the EGF receptor was not saturable with respect to the concentration of EGF used, the stimulation of fluid phase endocytosis was half maximal at an EGF concentration of 1 ng/ml and saturated at a concentration of 5 ng/ml. Also, the stimulation of fluid phase endocytosis was sevenfold greater initially after adding EGF than after a 30-min continuous incubation with the hormone, whereas the enhanced clearance of the EGF receptor did not change during this time period. We conclude that the EGF-stimulated increase in fluid phase endocytosis is not directly coupled to EGF-stimulated endocytosis of its own receptor but instead to a separate signal generated at the cell surface.

Key words: epidermal growth factor, receptors, endocytosis, cell surface, response kinetics, compartmentation

The addition of the polypeptide hormone epidermal growth factor (EGF) to cultures of responsive cells stimulates a wide range of cellular responses, including

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an increase in protein phosphorylation [1,2], amino acid uptake [3], fluid phase endocytosis [4], phospholipid turnover [5], and DNA synthesis and cell division [6,7]. How a single factor can modulate such diverse cellular activities is a central issue in determining the mode of action of hormones and growth factors. Postulated mechanisms include multiple classes of hormone receptors [8], an enzymatically active hormone–receptor complex with multiple substrates [9], and the existence of cellular "cascade" pathways that can be activated at a single locus [1]. In addition, it has been demonstrated that hormone–receptor complexes can be internalized by responsive cells, leading to suggestions that sites of signal generation can exist both at the cell surface and intracellularly [10,11]. Thus multiple effects of a hormone could be due to the presence of the hormone–receptor complex in multiple cellular compartments.

Recently we presented a set of steady state equations that acurately describe the binding, internalization, and degradation of EGF by human fibroblasts [12]. The primary aim in developing these equations was to facilitate quantitative evaluations of the relationships between specific interactions of the hormone with cells and specific cellular responses. We found that internalization of the EGF–receptor complex was extremely rapid (halflife on the cell surface of less than 5 min) and that greater than 85% of the EGF that was associated with human fibroblasts at steady state was present in an intracellular compartment [12]. These observations are particularly relevant to arguments relating early effects of EGF (such as increased amino acid transport) to cell surface events. It has been suggested that early effects of EGF could be due to its action at the cell surface since the observed time course of their induction (15 min to 1 hr) was thought to precede significant hormone internalization [13]. However, these hypotheses must be reevaluated in light of the observed time course of EGF internalization.

A major aspect of our research is to define the cellular compartments from which specific cellular responses are controlled. Identification of these compartments should greatly facilitate efforts to identify and isolate the structures and molecules that give rise to specific hormonal signals. In this study, we attempted to identify the cellular compartment responsible for increased fluid phase endocytosis induced by EGF. We chose this particular response because it is one of the most rapid EGF-induced cellular changes and also because it is clearly associated with the cell surface [4]. Thus it seemed likely that it was tightly coupled to occupancy of the EGF receptor. To accurately measure this reseponse, we developed an improved method for quantitating fluid phase endocytosis that has a time resolution of less than 1 min. These studies allowed us to conclude that the EGF-induced signal that gives rise to increased fluid phase endocytosis is generated at the cell surface.

MATERIALS AND METHODS

General

Mouse EGF was purified from submaxillary glands according to the method of Savage and Cohen [14]. This material gave a single band upon native polyacrylamide gel electrophoresis [15]. EGF was iodinated by the Iodogen method as previously described [15]. The specific activity of the preparation used in this study was 250,000 dpm/ng.

The human foreskin fibroblasts were prepared and cultured as described previously [12,15]. Strain SW-8 was used throughout this study. They were maintained in

Dulbecco's modified Eagle's medium (DV medium) from Flow Laboratories containing 10% calf serum. Cultures were plated at a density of 3×10^5 cells per 60-mm plastic dish and grown to a density of $0.9–1.2 \times 10^6$ cells per dish for use in experiments.

The binding of ^{125}I-EGF to cells was performed as previously described [12,15]. The value of the endocytotic rate constant (k_e) was determined using the computer-assisted method previously described [15]. All binding studies were conducted at 37°C in DV medium containing 1 mg/ml bovine serum albumin. Cell surface-bound ^{125}I-EGF was discriminated from internalized ^{125}I-EGF using our modification of the procedure of Haigler et al [15,16].

The integral of the number of occupied EGF receptors on the cell surface with respect to time was calculated using our computer-assisted method previously described [12]. This numeric integration routine is written in Basic for an Apple II computer and is available on request. The values for the cellular parameters used in this integration are as follows: $k_e = 2.35 \times 10^{-3}$ sec^{-1}, $k_r = 4.77 \times 10^{-5}$ sec^{-1}, $k_a = 2.9 \times 10^6$ molar^{-1} sec^{-1}, $k_d = 1.24 \times 10^{-2}$ sec^{-1}. Vr = 3.84 receptors per cell per sec, $k_h = 1.46 \times 10^{-2}$ min^{-1}, and a degradation lag time of 15 min. The concentrations of EGF, the number of cells and the volume of medium used in the integration program were the same as those in the experiments used in the comparisons.

Preparation of ^{125}I-PVP

^{125}I-labeled polyvinylpyrrolidone (^{125}I-PVP) was synthesized by a modification of the method of Briner [17]. Because of the large amount of radioiodine used in its synthesis (10 mCi), all operations were performed in an approved fume hood. The PVP (M_r 40,000, pharmaceutical grade) was obtained from Sigma. PVP solution (500 μl; 200 mg/ml in 0.2 N sulfuric acid) was cooled to 0°C in a microfuge tube. After adding 20 μl of ice cold 10% sodium nitrite and 400 μl of water, the contents were mixed and placed on ice for 10 min. The mixture was split into two equal aliquots and added to 6×0.35 cm ID quartz tubes containing a 1.5×15-mm magnetic stir bar. These tubes were modified so they could be capped with a screw threaded cap with a silicon rubber Teflon liner (Wheaton Glass Company, part #240581). To each tube was added 50 μl of carrier-free ^{125}I (Amersham 100 mCi/ml). The tubes were then taped horizontally by their caps onto an aluminum foil-covered magnetic stirring platform. An ultraviolet light source (254 nm, model UVS-11 Mineralight) was then placed less than 1 cm from the tubes. The solutions were irradiated and mixed for 48 hr. Then 50 μl of 10 mg/ml sodium sulfite in 5 N NH_4OH was added to each tube with mixing. The solutions were pooled and placed over a small (~3 ml) column of Dowex AG1-X8 (Cl-form) prewashed in water. The ^{125}I-PVP was then eluted with several washes of water. The ^{125}I-PVP was dialyzed overnight at 20°C against 2 liters of 0.75 M NaCl in 20 mM Na phosphate (pH 7.2) and then dialyzed at 4°C against two changes of DV lacking bicarbonate but buffered with 12.5 mM Hepes (pH 7.4). The ^{125}I-PVP solution was then diluted to 10 ml with DV medium and sterilized by filtration prior to storage at −20°C.

The specific activity of the ^{125}I-PVP was ~20 μCi/mg (20% coupling efficiency of the iodine) and was greater than 99% precipitable by 10% trichloroacetic acid.

Analysis of ^{125}I-PVP Uptake by Cells

Uptake studies were performed in Hepes-buffered DV medium containing 1 mg/ml bovine serum albumin. Cells were switched to this medium for at least 4 hr

prior to the uptake studies. Uptake of ^{125}I-PVP was initiated by switching the cells to prewarmed medium (37°C) containing the indicated concentrations of ^{125}I-PVP. At the specified times, the ^{125}I-PVP solution was removed and the plates were rinsed six times at 0°C with HP saline (130 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mg/ml PVP, 20 mM Hepes, and NaOH to give a pH of 7.5). The cells were allowed to remain in the last rinse for 15 min. The rinse solution was aspirated and then 1 ml of 0.05% trypsin in phosphate buffered saline containing 1 mM EDTA was added. After a 5-min incubation at 37°C, the detached cells were transferred to a microfuge tube and and a 100 μl aliquot was removed for determination of cell number using a Coulter Counter. The remaining cells were centrifuged in a Beckman Microfuge for 2 min. The trypsin solution was aspirated and the tips of the microfuge tubes containing the cell pellet were sliced off with a razor blade. Radioactivity in the pellets was then measured in a gamma counter. All uptake data were converted to femtoliters per cell.

RESULTS

EGF Stimulates Fluid Phase Endocytosis in Human Fibroblasts

Previous studies have demonstrated that EGF can stimulate fluid phase endocytosis in human A431 cells [4]. These transformed cells have been widely used in studies on EGF action since they possess very large numbers of EGF receptors (2–3 × 10^6 receptors/cell). However, it has not been demonstrated that EGF can stimulate fluid phase endocytosis in nontransformed human cells. To address this point, the effect of EGF on fluid phase endocytosis in normal fibroblasts was determined by measuring the uptake of ^{125}I-PVP, an inert polymer that has been demonstrated to be internalized by cells entirely by fluid phase endocytosis [18–20]. Cells were incubated in a fixed concentration of ^{125}I-PVP either in the presence or absence of 5 ng/ml EGF. At 5-min intervals the cells were evaluated for internalized radioactivity; the results are shown in Figure 1. The untreated cells demonstrated a linear time course of ^{125}I-PVP uptake as expected for a fluid phase endocytosis marker. The observed endocytotic rate (30 nl/10^6 cells/h) was in good agreement with previously reported values for fluid phase endocytosis in fibroblasts [21,22]. We found that the incorporation rate for ^{125}I-PVP remained constant for at least 12 hr in control cells (results not shown). In contrast, the EGF-treated cells demonstrated a burst of endocytotic activity for the first 10 min after EGF addition (Fig. 1). Thereafter, the rate of fluid uptake by the EGF-treated cells stabilized at a rate about 40% higher than the control cells. This initial burst of fluid uptake stimulated by EGF in human fibroblasts is very similar to the burst of fluid uptake observed by Haigler et al in A431 cells [4]. However, those investigators found that the fluid uptake rate in A431 cells quickly returned to basal values after the initial burst in contrast to the situation with HF cells (Fig. 1). To determine if an increased fluid phase endocytosis in HF cells persists after the initial 15-min burst, we preincubated cells either with or without 5 ng/ml EGF for 8 hr prior to measuring the rate of ^{125}I-PVP uptake in the presence or absence of EGF. As shown in Figure 2 cells that were pretreated with EGF did not demonstrate an initial burst of fluid uptake. However, they did have an enhanced rate of ^{125}I-PVP uptake that in this experiment was about 35% higher than the control cells. Since the pretreated cells were at steady state with regard to binding, internalization, and degradation of EGF [12], we conclude that EGF persistantly stimulates an increase in fluid phase endocytosis in HF cells.

The Kinetics of Induction of Fluid Phase Endocytosis by EGF

Since the major aim of this study was to identify the cellular site from which the signal for increased fluid uptake is generated, we compared the kinetics of induction of ^{125}I-PVP uptake with the kinetics of EGF binding and internalization. HF cells were treated with a fixed concentration of ^{125}I-PVP in the presence or absence of EGF and were evaluated at 1-min intervals for internalized radioactivity. As shown in Figure 3, fluid uptake of the untreated control cells was linear for the 5-min time course studied. However, EGF-treated cells demonstrated a sigmodial curve of uptake (Fig. 3A). When the uptake data for EGF-treated cells was expressed as the percentage of ^{125}I-PVP internalized over control cells, the induction curve presented in Figure 3B was obtained. Induction of ^{125}I-PVP uptake was very rapid (within 1 min) and reached a plateau by 4 min. Subsequently, the magnitude of this response was reduced (see Fig. 1).

To relate this to the kinetics of EGF binding and internalization, we examined the binding of ^{125}I-EGF to HF cells during a 5-min interval. Surface-bound and internalized EGF were discriminated using a computer-assisted method previously described [15]. The results of this experiment are shown in Figure 4. Binding of ^{125}I-EGF to the surface of HF cells described a smooth curve that reached a plateau by 4 min. Internalization of ^{125}I-EGF was also very rapid and was significant by 1 min. However, the kinetics of the surface binding and internalization of ^{125}I-EGF were quite different. Significantly, the kinetics of induction of ^{125}I-PVP uptake closely paralleled that of the surface binding of EGF but not that of its internalization. We

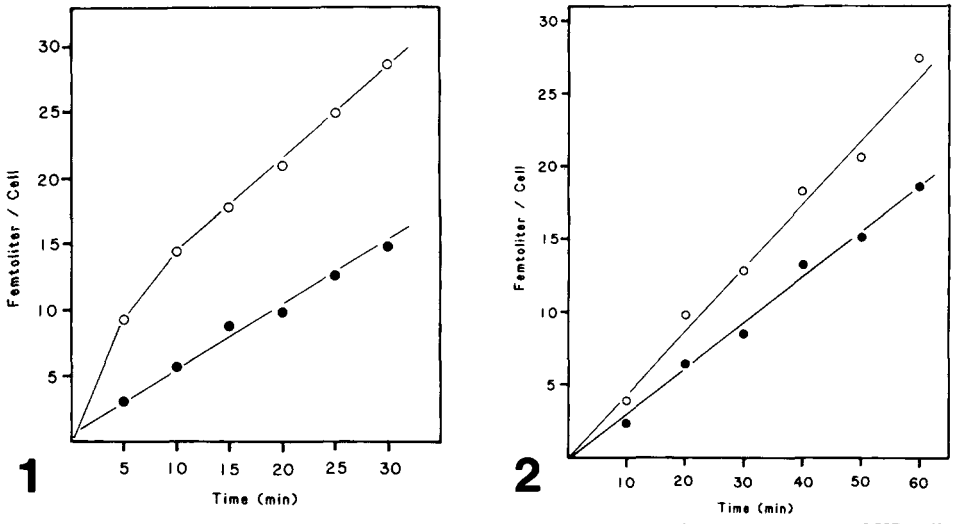

Fig. 1. Stimulation of fluid phase endocytosis by EGF in HF cells. Confluent monolayers of HF cells were incubated with ^{125}I-PVP in the presence (○) or absence (●) of 5 ng/ml EGF. At the indicated times the cells were assayed for the incorporation of radioactivity as detailed in Materials and Methods. The concentration of ^{125}I-PVP was 1.5×10^8 dpm/ml.

Fig. 2. Long-term stimulation of fluid phase endocytosis by EGF in HF cells. Confluent monolayers of HF cells were treated for 8 hr either with (○) or without (●) 5 ng/ml EGF prior to their exposure to ^{125}I-PVP. The cells pretreated with 5 ng/ml EGF also had the same concentration of EGF present during the assay for ^{125}I-PVP uptake. The amount of radioactivity incorporated was determined according to the procedure described in Materials and Methods. The concentration of ^{125}I-PVP was 6.1×10^7 dpm/ml.

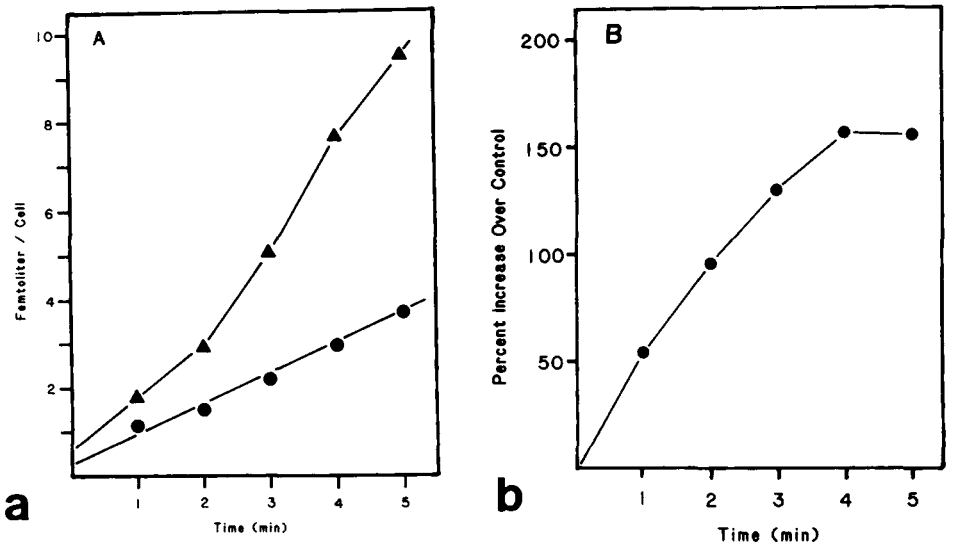

Fig. 3. The kinetics of induction of EGF-stimulated fluid phase endocytosis in HF cells. (A) The first five minutes of incorporation of ^{125}I-PVP by cells treated with (▲) or without (●) 5 ng/ml EGF. Confluent monolayers of HF cells were assayed for ^{125}I-PVP uptake at the indicated time intervals as described in Materials and Methods. The concentration of the ^{125}I-PVP was 1.5 × 10^8 dpm/ml. (B) The kinetics of induction expressed as percentage over control values. The data shown in panel A was converted to percentage over control values and plotted as a function of time.

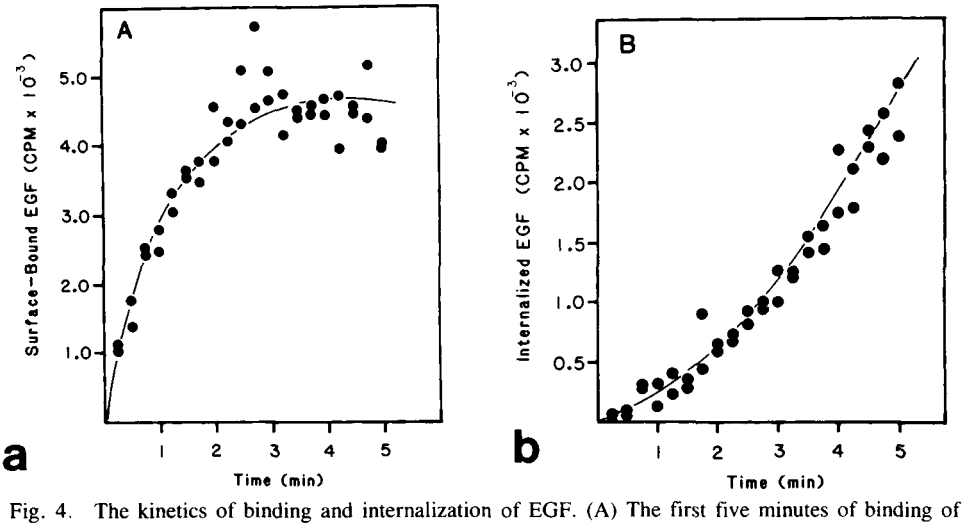

Fig. 4. The kinetics of binding and internalization of EGF. (A) The first five minutes of binding of EGF to the cell surface. Confluent monolayers of HF cells were exposed to 5 ng/ml of ^{125}I-EGF and at 15-sec intervals were assayed for the presence of surface-bound EGF as described in Materials and Methods. The data are the pooled results from two experiments and are corrected for nonspecific binding and spillover from the interior of the cell. (B) The first 5 min of EGF internalization by HF cells. These data were collected simultaneously with the data shown in panel A and are corrected for nonspecific binding and surface spillover. Note that there is no apparent lag between the binding of EGF to the surface of the cell and the internalization of the hormone.

conclude that the kinetics of induction of fluid phase endocytosis is consistent with that of a cell surface-mediated event.

Is the Enhanced Fluid Phase Endocytosis Related to Internalization of EGF?

The stimulation of fluid phase endocytosis by EGF in HF cells could be related to the phenomenon that the occupied EGF receptor in HF cells is removed from the cell surface greater than 14 times faster than the unoccupied receptor [12]. To determine the relationship, if any, between these two EGF-induced events, we examined their dependence on concentration of surface-bound EGF. Previously we demonstrated that the internalization rate of the EGF–receptor complex is directly proportional to its cell surface concentration [15]. To see if this relationship was also true of EGF-induced fluid phase endocytosis, cells were treated with a range of EGF concentrations and then tested for ^{125}I-PVP uptake rates. The results of this experiment are presented in Figure 5 together with data from a parallel set of cells tested for surface binding of ^{125}I-EGF. The stimulation of ^{125}I-PVP incorporation appeared to saturate at an EGF concentration of about 5 ng/ml. In contrast, surface binding of ^{125}I-EGF did not saturate until a concentration of greater than 25 ng/ml was reached (Fig. 5). However, the amount of ^{125}I-EGF measured on the cell surface at the end of the 15-min incubation period was a measure of the instantaneous value of binding while the amount of ^{125}I-PVP measured was the total amount internalized during the entire 15-min period. Thus it would be more valid to measure the relationship between the integral of the first 15 min of ^{125}I-EGF cell surface binding and the total ^{125}I-PVP incorporated. To perform this integration operation we used the computer simulation

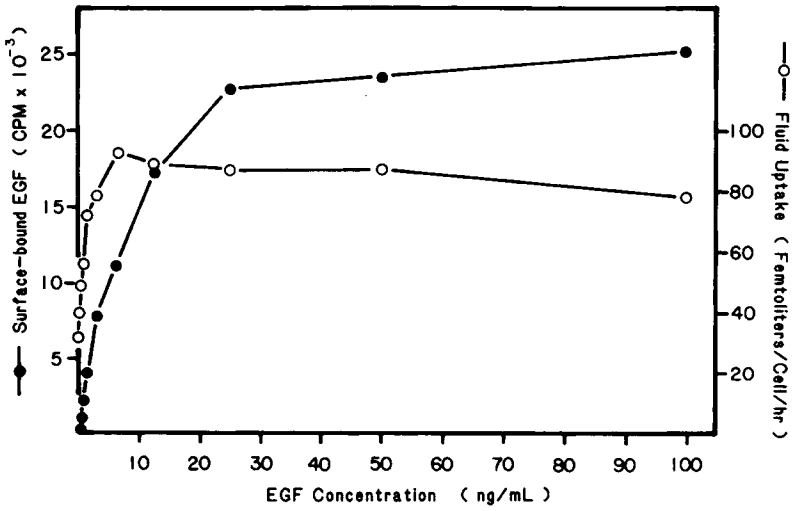

Fig. 5. The effect of EGF concentration on the stimulation of ^{125}I-PVP uptake and the binding of ^{125}I-EGF to the cell surface. Confluent monolayers of HF cells were incubated with ^{125}I-PVP and the indicated concentrations of unlabeled EGF for 15 min prior to assaying for incorporated radioactivity as detailed in Materials and Methods. The concentration of ^{125}I-PVP was 6.3 × 10^7 dpm/ml. The data (○) were converted to femtoliters incorporated per cell per hour. A parallel set of cells was incubated with the indicated concentrations of ^{125}I-EGF and 15 min later were assayed for cell surface-bound EGF (●) as detailed in Materials and Methods. The specific activity of the ^{125}I-EGF was 188,000 cpm/ng and the cell number was 7.9 × 10^5/60-mm dish.

of EGF binding and internalization described previously [12]. To test the validity of this computation, we also tested the relationship between the calculated integral of EGF cell surface binding and the amount of ^{125}I-EGF internalized by cells in 15 min. The results of these calculations are presented in Figure 6. There was a direct correlation between the calculated integral of the surface binding of EGF and its internalization. This is exactly as expected since we have shown previously that receptor occupancy is the only observed rate limiting step in EGF internalization [15]. However, we observed at least two rate limiting steps in the stimulated uptake if ^{125}I-PVP (Fig. 6). The binding of EGF was limiting at low levels of stimulated fluid uptake but other unknown processes became limiting at higher levels of fluid uptake. These results clearly demonstrate that the processes of EGF-stimulated fluid uptake and EGF internalization are not directly coupled.

It seemed possible that a limiting factor in the stimulate uptake of ^{125}I-PVP could be the time frame of the process since it seemed to be a transient response (see Fig. 1). However, that observation could have been due to "down regulation" of the EGF receptors from the cell surface. This posibility was tested by comparing the rate of increased fluid uptake stimulated by EGF to the time course of EGF receptor down regulation. The results of this comparison are shown in Figure 7. Although the total number of occupied EGF receptors decreased between 5 and 30 min of binding, there was a more rapid decrease in the stimulated rate of fluid uptake. This indicates that the transient nature of the EGF-stimulated fluid uptake was not due to down regulation of the receptor. Unlike the case of stimulated fluid uptake, EGF internalization was

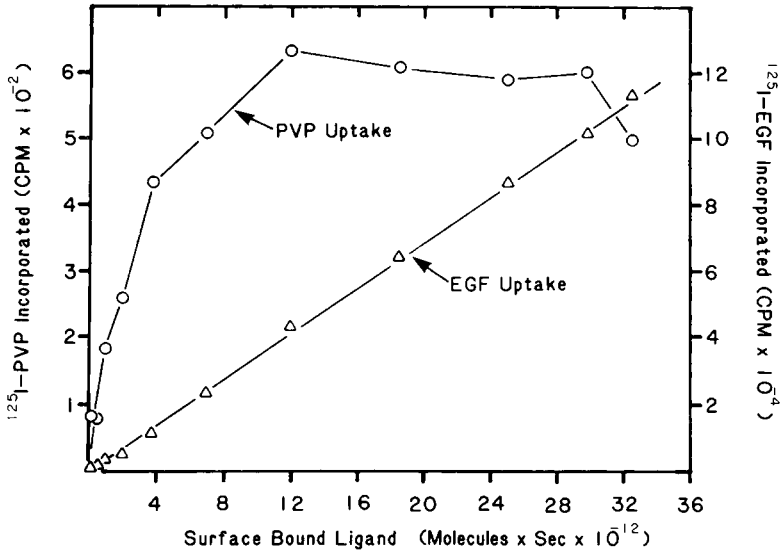

Fig. 6. A comparison between receptor occupancy, ^{125}I-PVP uptake, and EGF internalization. The integral of cell surface EGF receptors that were occupied at the EGF concentrations shown in Figure 5 was calculated using our computer model of the binding, internalization, and degradation of EGF as described in Materials and Methods. The time course integrated is the 15 min of the actual experiment. The calculated integral of occupied receptors was then compared with the amount of ^{125}I-PVP taken up during the experiment shown in Figure 5 and also with the amount of ^{125}I-EGF incorporated in a parallel set of cells. The ^{125}I-PVP incorporated is expressed as the amount that was taken up over the values in the cells not treated with EGF. All other conditions are the same as those described in Figure 5.

not a transient event. The rate of ^{125}I-EGF internalization was directly proportional to the amount of surface-bound ^{125}I-EGF from 3 min to 6 hr (rate constant of internalization of 0.16 min^{-1} at 3 min and 0.15 min^{-1} at 6 hr [15]). These results show that the time period of EGF internalization differs significantly from that of EGF-stimulated fluid uptake.

DISCUSSION

^{125}I-PVP was chosen as a marker to measure EGF-stimulated fluid phase endocytosis for several reasons. First, ^{125}I-PVP has been shown to absorb less to the cell surface than other markers of fluid phase endocytosis such as ^{14}C-sucrose [19,20], thus the interpretation of uptake studies is greatly simplified. In addition, ^{125}I-PVP is not degraded by cells subsequent to its incorporation (unlike horseradish peroxidase [22]), which also simplifies the interpretation of experiments. The presence of endogenous peroxidase-like activity in cells is another factor that limits the effectiveness of the horseradish peroxidase method for measuring fluid phase endocytosis in cells [4]. Unfortunately, ^{125}I-PVP is not commercially available in this country so we designed a small-scale method for labeling PVP. The relatively high specific activities obtained

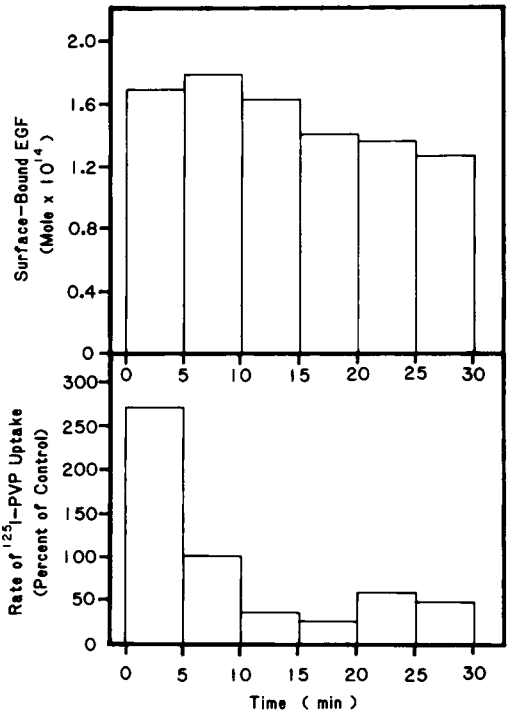

Fig. 7. A comparison between the time course of EGF-stimulated ^{125}I-PVP uptake and "down-regulation" of the EGF receptor. Confluent monolayers of HF cells were incubated in ^{125}I-PVP in the presence or absence of 5 ng/ml EGF. At 5-min intervals the rate of ^{125}I-PVP uptake was measured and is expressed as a percentage of the control value (lower panel). The upper panel shows the average amount of ^{125}I-EGF that was bound to the cell surface of a parallel set of cells during the indicated time intervals. The concentration of ^{125}I-EGF was 5 ng/ml and the cell number was 1.18 × 10^6 per monolayer. The amount of cell-surface bound EGF was determined as described in Materials and Methods.

(>20 μCi/mg) allowed us to examine cellular fluid uptake at a time resolution of 1 min using less than 10^6 cells. Thus it was feasible to examine the time course of induction of increased fluid phase endocytosis by EGF in HF cells.

The characteristics of the EGF-stimulated fluid uptake by HF cells is very similar to that observed by Haigler et al in A431 cells [4]. The initial burst of fluid uptake is over within 10 min and the maximum fluid uptake rate is stimulated by concentrations of EGF that are below the levels required to saturate the surface receptors. However, the magnitude of the response that we see with HF cells (approximately threefold) is less than that seen by Haigler et al in A431 cells (greater than tenfold). This may be due to the fact that we used a different method for measuring fluid uptake by cells (^{125}I-PVP versus horseradish peroxidase). In a series of unpublished experiments, we examined the effect of EGF on the rate of fluid phase endocytosis in A431 cells using ^{125}I-PVP as the fluid phase marker. The results obtained were qualitatively identical to those of Haigler et al [4]. However, we obtained only a threefold increase in the rate of fluid phase endocytosis in the first 15 min after adding EGF. Thus the response of HF cells and A431 cells may be identical with respect to this cellular parameter. This is somewhat surprising in light of the fact that A431 cells possess greater than 20 times the number of EGF receptors than HF cells.

All our results are consistent with the hypothesis [4] that the EGF–receptor complex generates a signal at the cell surface that results in an increased rate of fluid uptake. The response is very rapid and the time course of its induction closely parallels the time course of binding of EGF to the cell surface. This indicates that the two events are tightly coupled. It has been demonstrated previously that the time course of EGF-induced protein phosphorylation is also very rapid (<2 min) and thus is also probably coupled to cell surface events [1]. Since the EGF-stimulated protein kinase activity has been shown to be associated with EGF receptors partially purified by affinity chromatography [23], this result was not unexpected. However, EGF-stimulated fluid phase endocytosis has been shown to involve a significant change in the architecture of the cell surface, including the formation of new endocytotic vesicles [4]. Thus it is an example of a complex cellular event that is triggered rapidly at the cell surface by a growth factor-receptor complex.

The observation that EGF enhances the rate of clearance of EGF receptors from the cell surface by endocytosis and the observation that EGF enhances fluid phase endocytosis immediately after binding to its receptor would suggest a possible relationship between the two events. However, we could find no evidence for this. The EGF concentration dependence and the time course of stimulation of the two events were quite different. While the majority of the EGF-stimulated fluid uptake was transitory and reached a maximum by 5 min, EGF-stimulated receptor clearance was the same from 3 min to 6 hr. Likewise, the stimulation of EGF receptor clearance is directly proportional to the number of occupied surface receptors in HF cells [15] while the stimulation of fluid uptake in these cells clearly saturates at a level below full receptor occupancy (Fig. 6). Taken together, our results indicate that EGF-stimulated fluid uptake and the internalization of EGF are not directly coupled. This is not meant to imply that there is no relationship between the two events. It is obvious that occupancy of the EGF receptor is required for both responses. However, it would seem that the observed "down regulation" of the EGF receptor and the internalization of the hormone are not due to the stimulation of endocytosis per se. Since we observed

that the internalization of EGF had only a single rate limiting step while the stimulation of fluid uptake had at least two (Fig. 6), the only possible direct relationship that could exist is that the increased fluid uptake is a secondary result of the endocytosis of EGF. We feel that a more likely explanation is that the increased ^{125}I-PVP uptake and the internalization of EGF are two independent events triggered by the binding of the hormone to its receptor. The observed independent nature of these two responses (see Figs. 6 and 7) is certainly consistent with this hypothesis.

What then is the significance of the EGF-stimulated increase in fluid uptake? It is interesting to note that the characteristics of this response are very similar to the secretory response of human neutrophils to a chemotactic peptide. Sklar et al [24] have elegantly demonstrated that this response occurs at the cell surface due to receptor occupancy. It is a very rapid response (within 5 sec of exposure of the cell to the peptide) and is also transient. Furthermore, they demonstrate that the maximum response was elicited when no more than 20% of the cell surface receptors were occupied. Thus the characteristics of EGF-stimulated fluid uptake and the secretory response of neutrophils are very similar. It is possible that the observed increase in fluid uptake is simply due to the rapid retrieval of excess cell surface membrane that would be present after a secretory event. This could also explain the rapid membrane "ruffling" observed after the addition of EGF to A431 cells [25]. The ruffling could be due to the rapid insertion of membrane into the cell surface due to the secretory response while the enhanced fluid uptake would be due to a rapid retrieval of the excess membrane surface area. While we presently have no direct evidence for the coupling of these processes, the observed kinetics of these different events are consistent with their possible interrelationship.

The present work provides a set of criteria for evaluating the cellular site of action of EGF. As demonstrated in Figure 3B, there is no significant lag between the binding of EGF to its cell surface receptor and internalization of the EGF–receptor complex. Thus the stimulation of a cellular response within one hr after addition of EGF is not evidence that the site of action is at the cell surface. A much better criterion for the direct involvement of the cell surface is the demonstration that the kinetics of the induction of the response is the same as the kinetics of binding of EGF to its cell surface receptor. Certainly the delay of a response for one hr after addition of the hormone does not rule out the cell surface as the site of generation of the hormonal signal, but it is not supportive of such a site of action. However, differences in the time course of induction of various cellular responses by hormones is indicative of multiple mechanisms and pathways by which hormone–receptor complexes give rise to a variety of these responses. Analyses of the induction kinetics of various cellular responses could reveal which responses are coordinately controlled. Additionally, analyses of the kinetics of the interaction of hormones with different cellular compartments could provide insight into the sites of the cells from which different signals are being generated.

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