Sequential Appearance of Epidermal Growth Factor in Plasma Membrane-associated and Intracellular Vesicles during Endocytosis*

(Received for publication, April 28, 1987)

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Receptor-mediated internalization of epidermal growth factor (EGF) occurs by a process involving initially clathrin-coated pits on the cell surface and the subsequent formation of ligand-containing endosomes. Using a modified acid wash technique, cell surface-bound EGF was removed. Utilizing sucrose density centrifugation, the residual cell-associated EGF was separated into plasma membrane-associated and intracellular vesicle-associated forms. Using these procedures we have identified a transient form of cell-associated EGF that is still attached to the plasma membrane but not accessible to the extracellular fluid. This form of EGF appears to be the precursor for endosomal EGF. We suggest that this intermediate form represents the receptor-ligand complex shown by electron-microscopy to be located in narrow-necked plasma membrane invaginations (Willingham, M. C., and Pastan, I. (1980) Cell 21, 67–77).

Peptide hormones, such as epidermal growth factor (EGF),1 appear to enter cells by way of endocytosis (1–3). Electronmicrographs have revealed narrow-necked invaginations of the plasma membrane containing labeled cell surface receptors (4–6). These structures were proposed to represent an intermediate step in the transfer of receptor-ligand complexes between clathrin-coated pits on the cell surface and endosomes within the cell. We previously demonstrated (7) a possible biochemical correlate of such an intermediate step; specifically, the existence of two forms of intracellular EGF, one associated with plasma membrane (EGF<sub>pmv</sub>) and another associated with intracellular vesicles (EGF<sub>v</sub>). Further, acid-stable EGF appeared to accumulate more rapidly in the plasma membrane fraction than in the vesicular fraction. Here we attempt to determine if EGF<sub>pmv</sub> is formed and degraded independently of EGF<sub>v</sub> or, conversely, if it represents an intermediate stage in the formation of cytosolic vesicles. Narrow-necked structures, such as those observed by Pastan and co-workers (4–6) might provide a sequestered environment in which EGF could not be dissociated by a low pH wash but would still be attached to the plasma membrane. The results demonstrate that upon internalization EGF first appears in a plasma membrane-associated state and then is transferred to a vesicular fraction. We speculate that EGF<sub>pmv</sub> represents EGF in narrow-necked plasma membrane-associated invaginations.

**EXPERIMENTAL PROCEDURES**

**Materials—**EGF was obtained from Biomedical Technologies (Cambridge, MA) and sodium [125I]iodide from Amersham Corp. All other chemicals were purchased from Sigma.

**Cell Culture—**1321N1 human astrocytoma cells were grown in Dulbecco's modified Eagle's medium (low glucose) containing 5% fetal calf serum in a humidified incubator at 5% CO<sub>2</sub>. Cells were subcultured at a density of 10<sup>6</sup> cells/cm<sup>2</sup> 4 days prior to the experiment.

**EGF Iodination—**EGF was iodinated with chloramine T (8) to a specific activity of 0.5 Ci/mM EGF.

**Binding Assays—**[125I]EGF was diluted to a specific activity of 0.1–0.2 Ci/mM and used at a final concentration of 0.75 nM EGF. Binding was performed either at 37 or 4°C.

When binding was performed at 4°C, cells were first cooled in Hepes-buffered Eagle's medium containing 0.1% bovine serum albumin (HEB) for 30 min. [125I]EGF was then added for an additional 2 h. Cells were washed twice with HEB with 0.75 nM nonlabeled EGF added. Cells were incubated at 37°C for the time indicated, dishes were placed on ice, and cells were washed twice with HEB, incubated with 5 ml of 50 mM sodium acetate, pH 4.5 (2), or HEB for 5 min, washed twice with HEB, and treated for 20 min with 0.25 mg/ml concanavalin A. Cells were lysed by hypotonic shock, i.e. washed once with 1 mM Tris, 2 mM EDTA, pH 7.5, incubated for 20 min with Tri-EDTA, and scraped with a rubber policeman. Lysates were adjusted to 1 ml, layered on top of a sucrose step gradient (3.2 ml 15%, 4.2 ml 38%, 4.2 ml 60% sucrose), and centrifuged for 30 min at 35,000 rpm using a SW40 rotor. The interfaces were separated and filtered and radioactivity was determined. Plasma membrane accumulation at the 38%/60% sucrose interface (7); intracellular vesicles accumulated at the 15%/38% sucrose interface (7). When, prior to lysis, the cells were washed with sodium acetate, pH 4.5, the 38%/60% interface represents EGF<sub>pmv</sub> and the 15%/38% interface represents EGF<sub>v</sub>; when cells were washed with HEB, the 38%/60% interface represents total plasma membrane-associated EGF. EGF<sub>pmv</sub> was calculated as total plasma membrane-associated EGF minus EGF<sub>pmv</sub>.

Membrane-associated [125I]EGF in the two interfaces accounted for 95% of the total membrane-associated [125I]EGF on the gradient. When binding was performed at 37°C, cells were incubated with 5 ml of HEB containing 0.75 nM [125I]EGF. Dishes were then placed on ice, and cells were washed twice with HEB and incubated for 5 min with 5 ml of 40 mM sodium acetate, 150 mM NaCl, pH 5.1. Cells were washed twice with HEB and either kept on ice or incubated at 37°C in HEB with 0.75 nM EGF for the time indicated. Dishes were placed on ice, and cells were washed twice, incubated with concanavalin A, and further incubated as described above.

When total cell-associated EGF was determined, cells were incubated with [125I]EGF, dishes were placed on ice, and cells were washed twice with HEB, incubated for 5 min with the appropriate buffer, washed twice again with HEB, and solubilized in 0.1 N NaOH. Radioactivity was determined in a Beckman γ-counter.

**RESULTS AND DISCUSSION**

We have used two independent methods to measure the endocytosis of EGF. Others had shown that, whereas EGF bound to the cell surface is rapidly dissociated by washing with low pH buffers, internalized EGF remains cell-associated.

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1 The abbreviations used are: EGF, epidermal growth factor; EGF<sub>pmv</sub>, epidermal growth factor bound to cell surface receptors; EGF<sub>pmv</sub>, epidermal growth factor bound to receptors located in a plasma membrane-associated acid stable environment; EGF<sub>v</sub>, epidermal growth factor bound to receptors located in a light density vesicle; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HEB, Hepes-buffered Eagle's medium containing 0.1% bovine serum albumin.
EGF in Plasma Membrane-associated and Intracellular Vesicles

(2, 9). Exposure of cells to EGF at 37 °C leads to a time- and temperature-dependent formation of acid-stable (internalized) EGF. If cells are exposed to EGF at 4 °C, essentially all cell-associated EGF is acid-labile (cell surface). Upon warming to 37 °C, the portion of acid-stable EGF increases rapidly as endocytosis occurs.

EGF associated with the cell surface and that associated with intracellular membrane vesicles also can be distinguished using sucrose density centrifugation techniques (7, 10, 11). In this procedure cross-linking of cell surface proteins with canavanin A allows efficient separation of plasma membrane (identified by adenylate cyclase activity) and intracellular membrane vesicles due to their different densities (11). In general the two procedures produce agreeable results. However, in the early phase of the internalization process a substantial portion of the acid-stable EGF was found associated with the plasma membrane fraction (7). We have designated this form of EGF $\text{EGF}_{\text{PMV}}$.

The goal of the present work was to determine if EGF$_{\text{PMV}}$ serves as a precursor for the acid-stable EGF associated with intracellular membrane vesicles (EGF$_{\text{v}}$). Fig. 1 shows a simplified scheme of the proposed sequential pathway of EGF from the cell surface, EGF$_{\text{CS}}$, to plasma membrane-associated vesicles, EGF$_{\text{PMV}}$, and subsequently to intracellular vesicles, EGF$_{\text{v}}$.

**TABLE I**

**pH dependence of EGF receptor dissociation and ligand internalization**

In A, cells were incubated for 5 min at 37 °C in HEB containing 0.75 nM $^{125}$I-EGF. Culture dishes were then placed on ice, and the cells were washed, incubated for 5 min with 1 ml of the buffer indicated, washed, and solubilized in 1 ml of 0.1 N NaOH. In B, cells were incubated on ice for 5 min with the buffer indicated, washed, incubated for 20 min at 37 °C with HEB containing $^{125}$I-EGF, placed on ice, washed, incubated with 40 mM sodium acetate for 5 min, washed, and solubilized in 1 ml of 0.1 N NaOH. Experiments were done twice in triplicate, mean values ± S.D. are given.

| Buffer | Cell-associated EGF (mCi/dish) |
|--------|---------------------------------|
| A. Posttreatment varied |                                |
| HEB, pH 7.5 | 15.4 ± 0.3                     |
| 40 mM sodium acetate, pH 4.5 | 8.5 ± 0.9                      |
| 40 mM sodium acetate, 150 mM NaCl, pH 4.5 | 8.8 ± 0.6                     |
| 40 mM sodium acetate, pH 5.2 | 8.6 ± 0.7                      |
| 40 mM sodium acetate, 150 mM NaCl, pH 5.2 | 8.4 ± 1.1                     |
| 40 mM sodium acetate, pH 5.4 | 9.7 ± 0.4                      |
| B. Pretreatment varied |                                |
| HEB, pH 7.5 | 14.6 ± 0.7                     |
| 40 mM sodium acetate, pH 4.5 | 7.5 ± 1.1                      |
| 40 mM sodium acetate, pH 5.2 | 8.3 ± 0.3                      |
| 40 mM sodium acetate, 150 mM NaCl, pH 5.2 | 13.0 ± 1.3                     |

![FIG. 1. Scheme of internalization process.](image)

![FIG. 2. Time course of EGF appearance on the cell surface, in plasma membrane-associated and in free vesicles.](image)

![FIG. 3. Redistribution of EGF from plasma membrane-associated vesicles to free vesicles.](image)

EGF$_{\text{v}}$. The scheme indicates only those putative features of the overall pathway we focus on in this study. To determine if EGF$_{\text{PMV}}$ and EGF$_{\text{v}}$ are formed sequentially, it was necessary to determine if EGF$_{\text{v}}$ could still increase when cell surface acid-labile EGF (EGF$_{\text{CS}}$) was removed, and if under these conditions an increase in EGF$_{\text{v}}$ was accompanied by a decrease in EGF$_{\text{PMV}}$. Fig. 2 shows a detailed time course of the formation of EGF$_{\text{v}}$ and EGF$_{\text{PMV}}$, which were separated on a sucrose step gradient (7). Cells were exposed to $^{125}$I-EGF at 4 °C to allow binding to the receptor. Internalization occurred during a subsequent incubation at 37 °C in the presence of nonlabeled EGF. In one set of cultures surface-bound EGF was removed by a low pH wash (3), while in a parallel set cells were washed at neutral pH to measure the total amount of cell-associated EGF. EGF$_{\text{CS}}$ was defined as the difference in EGF associated with the plasma membrane fraction of the sucrose gradients from neutral and acid-washed cells. The
rate of loss of EGF\textsubscript{CS} and the rate of increase in EGF\textsubscript{V} and EGF\textsubscript{PMV} were determined from the data of two independent experiments, one of which is shown in Fig. 2.

EGF\textsubscript{CS} disappeared at a rate of 4.8 ± 0.2 fmol/10 min/dish reaching a nondetectable level within 8–10 min. Concomitantly, the amount of EGF\textsubscript{PMV} increased at a rate of 2.2 ± 0.9 fmol/10 min/dish reaching a maximum after approximately 8 min. The amount of EGF\textsubscript{V} increased at a rate of 0.9 ± 0.1 fmol/10 min/dish, reaching a maximum significantly later than EGF\textsubscript{PMV} at 25 min. The additional loss of EGF\textsubscript{CS} was due to concomitant dissociation of the receptor-ligand complex estimated at a rate of 1.6 fmol/10 min/dish \( (k_{\text{off}} = 7.3 \times 10^{-4}/s) \). The increase in EGF\textsubscript{V} between 8 and 25 min was associated with a decrease in EGF\textsubscript{PMV} (0.5 ± 0.1 fmol/10 min/dish), suggesting the latter as a source for EGF\textsubscript{V}. After reaching a plateau, vesicular EGF declined at a rate of 0.40 ± 0.05 fmol/10 min/dish due to extrusion of \textsuperscript{125}I-EGF and its degradation products. Similar experiments using slightly different wash or preincubation conditions confirmed these results. These experiments demonstrate that the amount of EGF\textsubscript{V} increases after the cell surface has been depleted of EGF.

The protocol described above involves separating the binding and internalization reactions by making use of the differential effects of reduced temperature on these reactions. However, the possibility exists that EGF\textsubscript{PMV} is an artifact of the solvent or preincubation conditions confirmed these results. These experiments demonstrate that the amount of EGF\textsubscript{V} increases after the cell surface has been depleted of EGF.

Under the standard conditions (2) used to dissociate the cell surface EGF receptor complex, i.e. hypotonic buffer, pH 4.5, subsequent internalization is inhibited (Table IB). Increasing the pH to 5.2 and using an isotonic buffer resulted in removal of cell EGF\textsubscript{CS} (Table IA) without preventing subsequent internalization of EGF\textsubscript{V}. The latter conditions were used to remove cell surface-bound EGF after different periods of incubation with EGF at 37 °C. The subsequent kinetics of appearance of EGF\textsubscript{PMV} and EGF\textsubscript{V} were studied. Fig. 3A (solid symbols) shows the time-dependent increase in EGF\textsubscript{PMV}, while iodo-EGF is continuously present (solid symbols). The overall kinetics are faster than in the experiment shown in Fig. 2, because the cells were not pretreated at 4 °C (see also Table I, A and B). Once EGF\textsubscript{CS} is removed (open symbols), the amount of EGF\textsubscript{PMV} decreases. Fig. 3B shows the rapid increase in EGF\textsubscript{V}. Furthermore, in contrast to EGF\textsubscript{PMV}, a further increase in EGF\textsubscript{V} is observed after depleting EGF\textsubscript{CS}. The results demonstrate that the formation of EGF\textsubscript{PMV} is dependent on the continuous presence of EGF on the cell surface, whereas EGF\textsubscript{V} can accumulate, at least transiently, in the absence of EGF on the cell surface. This additional increase in EGF\textsubscript{V} was most probably derived by transfer from EGF\textsubscript{PMV}.

The present communication describes results from two independent approaches that demonstrate a sequential translocation of surface bound EGF (EGF\textsubscript{CS}), first to a plasma membrane-associated acid-stable form (EGF\textsubscript{PMV}) and then to an intracellular vesicular form (EGF\textsubscript{V}). We speculate that EGF\textsubscript{PMV} reflects EGF in the narrow-necked plasma membrane invagination, which have been shown by electronmicroscopy (4–6).

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\textsuperscript{2} C. Hertel and H. Affolter, manuscript in preparation.