Ligand-mediated Autophosphorylation Activity of the Epidermal Growth Factor Receptor during Internalization

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Abstract. The association of EGF with its receptor in endosomes isolated from rat liver homogenates was assessed biochemically by polyethylene glycol precipitation and morphologically by electron microscope radioautography. The proportion of receptor-bound ligand in endosomes at 15 min after the injection of doses of 0.1 and 1 μg EGF/100 g body weight was 57%. This value increased to 77% for the dose of 10 μg EGF injected. Quantitative electron microscope radioautography carried out on endosomes isolated at 15 min after the injection of 10 μg 125I-EGF demonstrated that most radiolabel was over the endosomal periphery thereby indicating that ligand–receptor complexes were in the bounding membrane but not in intraluminal vesicles of the content. EGF receptor autophosphorylation activity during internalization was evaluated in plasmalemma and endosome fractions. This activity was markedly but transiently reduced on the cell surface shortly after the administration of saturating doses of EGF. The same activity, however, was augmented and prolonged in endosomes for up to 30 min after EGF injection. The transient desensitization of cell surface activity was not due to prior in vivo phosphorylation since receptor dephosphorylation in vitro failed to restore autophosphorylation activity. Transient desensitization of cell surface autophosphorylation activity coincided with a diminished capacity for endocytosis of 125I-EGF with endocytosis returning to normal after the restoration of cell surface autophosphorylation activity. The inhibition of cell surface autophosphorylation activity and the activation of endosomal autophosphorylation activity coincident with downregulation suggest that EGF receptor traffic is governed by ligand-regulated phosphorylation activity.

The EGF receptor contains intrinsic tyrosine kinase activity which appears to be active after internalization into endosomes (12, 20). EGF receptor tyrosine kinase activity has been postulated by Honegger et al. (19) to control downregulation. Substitution of the ATP binding Lys 721 of the receptor for Ala and subsequent transfection into receptor-deficient NIH 3T3 cells resulted in the absence of downregulation but the maintenance of receptor-mediated endocytosis of EGF. Hence, constitutive receptor recycling was proposed by these authors (19). The role of the tyrosine kinase domain of the EGF receptor in regulating internalization and traffic of the receptor has also been suggested by the mutational analyses carried out by Lin et al. (30), Prywes et al. (35), and Livneh et al. (31). Furthermore, Glenney et al. (18) have demonstrated that microinjection of anti-phosphotyrosine antibody into receptor-transfected Chinese hamster ovary cells inhibited ligand-mediated EGF receptor internalization. Sorting of incoming receptors and ligands is regulated within the endosomal apparatus (6, 21, 33, 40), and EGF receptor phosphorylation is ligand dependent. We have consequently examined the degree of association of EGF with its receptor in endosomes as a function of the dose of EGF injected. Furthermore, the autophosphorylation activity of the receptor was evaluated in endosomes and at the cell surface as a consequence of the dose of injected EGF. We have observed that the majority of internalized EGF in endosomes is receptor bound and probably responsible for the enhanced autophosphorylation activity of internalized EGF receptor kinase. We have also observed a transient desensitization of the autophosphorylation activity of the cell surface EGF receptor but only when saturating doses of ligand were administered. This transient desensitization of autophosphorylation activity coincided with a marked inhibition in receptor-mediated endocytosis of EGF in vivo.

Materials and Methods

Materials, animals, EGF iodination, binding assays, SDS-PAGE, and subcellular fractionation protocols were as described in the companion paper (29).
Evaluation of Receptor-bound EGF in Endosomes by Selective Precipitation

Several modifications of the polyethylene glycol (PEG) precipitation method of Cuatrecasas (14) were necessary to evaluate the proportion of EGF bound to receptor within isolated endosomes. Control experiments were carried out to assess the effects of solubilization by Triton X-100. Steady-state direct binding of 

\[ ^{125}I \text{-EGF} \] to plasmalemma (PM) and Golgi apparatus-endosome (GE) fractions were carried out as described previously with 30 ~g of protein of PM or GE fractions incubated in a final volume of 0.5 ml with 

\[ ^{125}I \text{-EGF} (75,000 \text{ dpm}, 180 \mu \text{Ci/ug sp act}) \] for 14 h at 4°C with constant shaking (27). Ligand binding was determined by filtration. Solubilization of cell fractions was assessed with increasing concentrations of Triton X-100 (at 0°C for 3 min) followed by filtration on Whatman filters (9). Receptor-ligand complexes were precipitated by the addition of 1 ml of 0.125% -globulin and 1 ml of 25% PEG 8000 for 30 min at 4°C as in the method of Cuatrecasas (14).

Modifications included the addition of Con A (1 mg/ml) or a mixture of the lectins (Con A, wheat germ agglutinin, and Lens culinaris lectin) (1 mg/ml each), based on the prior studies of Krupp et al. (25) and Yarden et al. (41). Tubes containing the receptor-ligand complexes were at 4°C and Con A or lectin mixtures were added (at 4°C for 30 min) followed by the addition of PEG and -globulin as described above. As documented in Results, the final protocol which was efficient in separating free ligand from receptor-bound ligand was 0.5% Triton X-100, -globulin, PEG, and the lectin mixture (concentrations as above) all added together with the cell fraction for 30 min at 4°C followed by filtration.

Evaluation of Receptor-bound EGF by Quantitative Radioautography

GE fractions were prepared 15 min after a portal vein injection of 

\[ ^{125}I \text{-EGF} (10 \mu g/100 g body weight, 7.5 \times 10^5 \text{ dpm}, 259 \mu \text{Ci/ug sp act}) \] and fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, at 4°C overnight. Aliquots containing 30 ~g of protein were filtered onto filters (MF type; Millipore Continental Water Systems, Bedford, MA) under N2 night. Aliquots containing 30 ~g of fraction protein were filtered onto filters and samples were dephosphorylated as described above. The filters were processed for electron microscope radioautography as described previously (1). Con A or lectin mixtures were added (at 4°C for 30 rain) followed by the addition of PEG and -globulin and 3, -globulin as described above. As documented in Results, the final protocol which was efficient in separating free ligand from receptor-bound ligand was 0.5% Triton X-100, -globulin, PEG, and the lectin mixture (concentrations as above) all added together with the cell fraction for 30 min at 4°C followed by filtration.

EGF Receptor Autophosphorylation Activity and SDS-PAGE

EGF receptor autophosphorylation activity was evaluated on endosomal profiles included in the sample (average diameter 320 nm) as outlined by Salpeter et al. (37). Briefly, samples were phosphorylated and terminated with 10 ~l of 50 mM ATP in a 3:1 ratio (vol/vol).

Phosphatase Preparation

The liver cytosolic fraction which was used as a source of tyrosine phosphatase activity was prepared in 0.25 M sucrose solution with protease inhibitors (1 mM benzamidine, 50 ~M pepstatin, 1 mM PMSF, and 1,000 kalrieken inhibitor units/ml aprotinin). The homogenate was adjusted to 20% of 0.25 M STM (0.25 M sucrose, 5 mM Tris, pH 7.4, 1 mM MgCl2) with constant shaking at 4°C. Samples were centrifuged at 100,000 g for 30 min, and supernatants were processed for SDS-PAGE.

EGF Receptor Dephosphorylation and Rephosphorylation

Samples were phosphorylated and terminated with 10 ~l of 50 mM ATP in 20 mM Hepes, pH 7.5. Dephosphorylation was initiated with the addition of 200 ~l of freshly prepared cytosolic phosphatase at 22°C for 30 min, the reaction was stopped by adding equal volumes of 2X concentrated SDS-PAGE sample preparation buffer, and the samples were boiled. The rephosphorylation assay was carried out as follows. Aliquots of PM were incubated with equal volumes of cytosolic phosphatase at 22°C for 30 min, and samples were dephosphorylated as described above. At the end of dephosphorylation, the reactions were terminated by the addition of protease inhibitors or containing protease inhibitors plus the phosphatase inhibitors (0.8 mM ZnSO4, 80 ~M sodium vanadate, 160 mM sodium molybdate, 56 mM sodium fluoride, 160 mM \( \beta \)-glycerophosphate) in a 3:1 ratio (vol/vol).

Results

Conditions for Estimation of Ligand–Receptor Complexes in PM and GE Fractions

Control experiments were carried out to evaluate the retention of ligand–receptor complexes in PM after solubilization with Triton X-100. Direct binding of 

\[ ^{125}I \text{-EGF} \] to PM was provided by Dr. C. E. Chandler (Stanford University, Stanford, CA) and was subcloned (IgG-151, BH-6) by Drs. W. Dunn and A. L. Hubbard (The Johns Hopkins University, Baltimore, MD). Antibodies were isolated from cell culture supernatants (11) or from ascites fluid (34) and were purified by protein A-Sepharose chromatography. Control experiments showed that IgG-151 was a competitive inhibitor of 

\[ ^{125}I \text{-EGF} \] binding as well as for EGF-stimulating receptor kinase activity in both PM and GE fractions from rat liver (data not shown). The antibody was not, however, useful for quantitative immunoblotting.
Figure 1. Evaluation of conditions for estimating ligand–receptor complexes for EGF in PM (A) and GE fractions (B) and the demonstration of ligand–receptor association in isolated GE fractions (C). (A and B) Direct binding of 125I-EGF under steady-state conditions to intact PM and GE fractions was carried out as described in the companion paper (29) and normalized to 100%. Membranes were then evaluated for solubilization by increasing concentrations of Triton X-100 alone (o) as assessed by filtration. For each concentration of Triton X-100, the retention of ligand–receptor complexes was assessed by PEG precipitation with γ-globulin followed by filtration (A). This was compared with the retention of complexes after a 3-min solubilization in Triton X-100 followed by the addition of lectin mixtures followed by PEG with γ-globulin together (●). This is also compared with conditions where Triton X-100, lectins, PEG, and γ-globulin were all added together (■). Only under the latter conditions were ligand–receptor complexes maintained in solubilized (0.5% Triton X-100) PM or GE fractions. (C) Degree of receptor–ligand association in GE fractions as a function of the dose of EGF injected. At 15 min after the injection of 0.1, 1, and 10 µg of 125I-EGF, GE fractions were isolated and the proportion of free and receptor-bound ligand estimated by the addition of a mixture of Triton X-100, lectins, PEG, and γ-globulin (mean of three experiments ± SD and each determination was carried out in triplicate).

carried out at steady state. As shown in Fig. 1 A, the addition of Triton X-100 alone to PM with receptor-bound 125I-EGF led to the loss of 50% of radiolabel to the soluble fraction at a concentration of 0.18% Triton X-100. Solubilization of PM occurred with 1% Triton X-100 at which only 18% of radiolabel was retained by filtration. Subsequent attempts to precipitate solubilized ligand–receptor complexes with PEG and γ-globulin alone were unsuccessful. Indeed, the addition of PEG and γ-globulin 3 min after Triton X-100 solubilization appeared to promote ligand dissociation from receptor since 50% of radiolabel was not retained by filtration when the concentration of Triton X-100 was only 0.08% and only 14% of radiolabel was retained at 1% Triton X-100. The addition of a cocktail of lectins along with PEG 3 min after solubilization led to more complete retention of receptor-bound EGF, with 57% of the radiolabel retained at concentrations as high as 1% Triton X-100. Only with a protocol in which Triton X-100, PEG, and the lectin cocktail were added together could solubilization be effected with complete retention of receptor-bound ligand (Fig. 1 A). The use of the same conditions to evaluate the retention of EGF bound to its receptor in GE fractions showed a similar response to Triton X-100 alone and to the addition of PEG 3 min after Triton X-100 (Fig. 1 B). However, in contrast to the EGF receptor of PM, the addition of PEG plus lectins 3 min after solubilization with Triton X-100 was as efficient in maintaining ligand–receptor association as the addition of the PEG plus lectin cocktail plus Triton X-100. The differences between the data of Fig. 1, A and B, related only to conditions where the lectins were added 3 min after Triton X-100 and may reflect differences in the aggregation state of glycoconjugates (lectin-binding sites) found between PM and GE fractions.

The degree of ligand–receptor association in isolated GE fractions was assessed as a function of the dose of EGF injected into rats. Under conditions that solubilized the fractions with minimal EGF dissociation — i.e., with the cocktail of Triton X-100, lectins, γ-globulin, and PEG — ~57% of 125I-EGF in endosomes was receptor bound at doses of EGF injected of 0.1 and 1 µg of 125I-EGF/100 g body weight. The value increased to 77% receptor bound at 10 µg EGF injected (Fig. 1 C).

The degree of ligand–receptor association in the GE fraction was evaluated further by the technique of quantitative electron microscope radioautography. 15 min after the injection of 10 µg 125I-EGF, GE fractions were isolated and processed for radioautography. Silver grains overlaid liver endosomes with their characteristic intraluminal content of lipoprotein-like particles (Fig. 2). Qualitatively, the silver grains seemed to be at the periphery of endosomes. Evaluation of the distribution of grain densities within and outside of endosomes (Fig. 3) showed a peak over the endosomal membrane. Grain densities decreased markedly as a function of distance outside the endosomal profile and also decreased (although less so) as a function of distance within the endosome.

As proposed by Salpeter et al. (37), consideration of the
half-distance of $^{125}\text{I}-\text{EGF}$ (80 nm in our hands [5]) and the diameter of the vesicular profiles under analysis allows one to calculate the expected distribution of grain densities whether the source of radiolabel was free in the lumen or exclusively membrane bound. Such a calculation showed that the distribution expected for a membrane-bound source (Fig. 3, dashed line) closely approximated the observed distribution of grain densities (histogram). The limitation of this analysis was such that only endosomal profiles of diameter $>200$ nm could be included in the study. At diameters less than this, the distribution of silver grains expected for a uniform intraluminal source of radioactivity became very similar to that for a membrane source (37). These smaller vesicles, however, accounted for only 35% of the total silver grains; therefore, the majority of $^{125}\text{I}-\text{EGF}$ in the endosome preparation was subjected to the analysis of Fig. 3. Comparison of the empirically determined grain densities with the calculated distribution of grain densities indicated that $>99\%$ of the silver grains analyzed were from a radiolabeled source at the periphery of endosomes of the GE fraction. The radioautographic technique was impractical for lower doses of $^{125}\text{I}-\text{EGF}$ injected (0.1 and 1 $\mu$g) since the number of silver grains were too few for statistical analysis.

**Autophosphorylation Activity and Receptor Recycling**

The intraendosomal association of EGF with its receptor at the bounding membrane of endosomes prompted the study of changes in autophosphorylation activity during internalization and recycling. Autophosphorylation studies were carried out on intact and solubilized fractions in the presence or absence of in vitro-saturating concentrations of EGF to evaluate the endogenous autophosphorylation activity as a function of the maximal stimulated activity. (Electron microscopy showed no difference in the morphology of GE fractions after autophosphorylation assays carried out in the absence of detergent as compared with freshly prepared GE fractions.) As our past study has shown (20), most of the autophosphorylation of the EGF receptor under these conditions was on tyrosine residues. Therefore, evaluation of autophosphorylation was carried out after alkali treatment of gels (13). This simplified the phosphopeptide pattern enabling the ready observation of the band at 170 kD corresponding to the EGF receptor (Fig. 4). This protein was identified further as...
the EGF receptor by immunoprecipitation with the monoclonal antibody IgG-151, BH-6 (see below). Visual analysis of the intensity of labeling of the EGF receptor in PM at 15 and 120 min after the in vivo injection of 1, 5, and 10 μg EGF/100 g body weight showed a decrease at 15 min, the magnitude of which was larger as the amount of EGF injected was increased. By 120 min, 32P-labeled receptor was more intensely labeled than at 15 min for each dose of EGF injected (Fig. 4).

No lag preceded the loss of autophosphorylation activity from the PM or its accumulation into GE fractions (Fig. 5). Rates of loss of autophosphorylation activity from the PM were rapid and increased progressively from 0.15 min⁻¹ for 0.5 μg EGF injected to 0.9 min⁻¹ at 10 μg EGF injected under the in vitro assay conditions of maximal stimulation (with EGF and Triton X-100 in vitro). The rate of entry of the activity into GE fractions was correspondingly rapid (Fig. 5).

Augmentation of autophosphorylation activity by in vitro EGF was observed for PM-located receptor. However, at high doses of EGF injected (5 and 10 μg), autophosphorylation activity was nearly completely inhibited on the PM at 15 min after injection in confirmation of our past study at even higher doses of EGF injected (20). Also, as reported previously by us (20) and confirmed here, high doses of injected EGF (i.e., 10 μg) appeared to activate maximally autophosphorylation activity on the GE fraction whether or not EGF was added in vitro. At lower doses injected (0.1-1 μg), autophosphorylation activity was augmented by in vitro EGF added to GE fractions providing that Triton X-100 was present in the incubation mixture.

Immunoprecipitation studies (Fig. 6) confirmed the marked changes in autophosphorylation activity observed on the PM. To evaluate the contribution of newly synthesized protein to the autophosphorylation activity observed at 120 min, experiments were also carried out on fractions isolated from cycloheximide-injected rats. Quantitation (Fig. 7) confirmed the marked inhibition of autophosphorylation at 15 min after the injection of high doses of EGF (5 and 10 μg) and the reappearance of autophosphorylation activity at 120 min, which was least at the highest dose of injected EGF (10 μg).

**EGF Receptor Dephosphorylation and Rephosphorylation**

Autophosphorylation activity at 15 min after the injection of saturating doses of EGF (5 and 10 μg) was almost zero in PM fractions (Figs. 5 and 7) whereas ~25-40% of EGF receptors, as evaluated by direct binding or immunoblotting, remained on the cell surface (see Figs. 1, 3, and 5 of the companion paper [29]). This apparent desensitization of autophosphorylation activity was evaluated further. PM fractions were isolated at 15 min after the injection of a saturating dose of EGF. We then attempted to dephosphorylate the membranes in the presence and absence of EGF in vitro.

As shown in Fig. 8 A, receptor autophosphorylation in PM isolated from control (uninjected) rats (lanes 1 and 3) or 15 min (lanes 2 and 4) after EGF injection showed EGF-dependent autophosphorylation activity only for the PM isolated from uninjected rats (lane 3). Fig. 8 B demonstrated...
that the EGF receptor could be dephosphorylated by the phosphatase activity of liver cytosol (lanes 5, 6, 9, and 10). This dephosphorylation was inhibited by the addition of phosphatase inhibitors (lanes 7, 8, II, and J2). However, the phosphatase inhibitors appeared to be only partially effective as the level of phosphorylation appeared slightly lower than controls (i.e., lanes 1–4). Further experimentation showed that the dephosphorylated receptor could then be rephosphorylated but only back to near original (control; lanes 1–4) levels (Fig. 8C, lanes 13–20).

**Desensitization of Autophosphorylation Coincides with Diminished Internalization**

We evaluated ligand internalization at 15 min after the injection of saturating doses of EGF — i.e., when autophosphorylation activity was minimal. The experimental design consisted of injecting 0.1, 1, and 10 μg 125I-EGF at 10 min after the previous injection of 0.1, 1, and 5 μg of unlabeled EGF. Rats were then killed 15 min later and the GE fraction was isolated. The results (Fig. 9, solid bars) showed a high proportion of ligand internalized at the 0.1- and 1-μg doses but not at the 5-μg dose. These observations were compared with control experiments where 0.1, 1, and 10 μg 125I-EGF were injected into control (uninjected) rats, and the GE fraction was isolated 15 min later (open bars). An additional control was designed to account for any changes in specific radioactivity of the 125I-EGF due to the possibility of dilution in the circulation of previously injected unlabeled EGF. Hence, rats were injected with either a mixture of 0.1 μg unlabeled EGF plus 0.1 μg 125I-EGF; 1 μg unlabeled EGF plus 1 μg 125I-EGF; or 5 μg unlabeled EGF plus 10 μg 125I-EGF. GE fractions were isolated 15 min later (slashed bars). Extensive internalization of 125I-EGF was observed for both sets of controls (Fig. 9).

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**Figure 5.** Dose dependency of changes in autophosphorylation activity in PM and GE fractions. Autophosphorylation activity per unit cell fraction protein is shown for conditions of incubations carried out in the presence of 0.05% Triton X-100 with (●) or without (○) EGF in vitro. Similar findings were observed in the absence of Triton X-100 except for latency in endosomes (20).

**Table:**

| EGF injected (μg/100g bw) | 0   | 0.1 | 0.1 | 1   | 1   | 5   | 10  |
|----------------------------|-----|-----|-----|-----|-----|-----|-----|
| Time after injection (min) | 0   | 15  | 120 | 15  | 120 | 15  | 120 |

| EGF injected (μg/100g bw) | 0   | 0.1 | 0.1 | 1   | 1   | 5   | 10  |
|----------------------------|-----|-----|-----|-----|-----|-----|-----|
| Time after injection (min) | 0   | 0.1 | 0.1 | 15  | 15  | 15  | 15  |

**Figure 6.** Immunoprecipitation of EGF receptor kinase of PM after in vitro autophosphorylation. At 0, 15, and 120 min after EGF injection, PM was isolated from untreated (A) or cycloheximide-treated (B) rats, and autophosphorylation was carried out. EGF receptor kinase was immunoprecipitated with monoclonal antibody IgG-151, BH-6. The immunoprecipitate was electrophoresed, and alkali-treated gels were exposed to x-ray film for 1.5 d. Lanes 1 in A and B refer to immunoprecipitations carried out with rabbit antismouse IgG instead of monoclonal antibody IgG-151, BH-6. Each lane represents an immunoprecipitation carried out on 6 μg PM protein.
A further ligand internalization study was carried out at 60 min after the injection of EGF – i.e., when autophosphorylation activity was restored. At this time interval, the receptor content of the PM fraction had returned to near initial values for the 0.1- and 1-μg doses of injected EGF but remained downregulated at the 10-μg dose (29). Tracer doses of 125I-EGF were injected at 60 min after the injection of 0.1, 1, and 10 μg EGF/100 g body weight. The kinetics and extent of 125I-EGF accumulation in the GE fraction was evaluated (Fig. 10) and found to be near identical.

Figure 7. In vivo dose response of autophosphorylation activity in PM without EGF injection (0 min) and 15 and 120 min after the injection with 0.1 (○), 1 (△), and 10 μg EGF (■). After autophosphorylation (with EGF and Triton X-100 in vitro), EGF receptor was immunoprecipitated and subjected to SDS-PAGE and radioautography, and the incorporated radioactivity quantified by densitometry. The number of experiments is indicated in parentheses.

Figure 8. EGF receptor protein autophosphorylation, dephosphorylation, and repolysis in PM. The radioautograph shows autophosphorylation activity with control PM fractions (without EGF injection; lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, and 19) and in PM fractions isolated at 15 min after the injection of 10 μg/100 g body weight EGF (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20). (A) Autophosphorylation of the EGF receptor. PM was isolated and incubated in the standard autophosphorylation assay as described in Materials and Methods in the absence (lanes 1 and 2) and the presence (lanes 3 and 4) of in vitro EGF. (B) Dephosphorylation by cytosolic phosphatase. Dephosphorylation was carried out after prior autophosphorylation without EGF in vitro (lanes 5–8) and with EGF in vitro (lanes 9–12), and the samples were electrophoresed as described in Materials and Methods. Controls were carried out in which the dephosphorylation reaction was carried out in the presence of phosphatase inhibitors (lanes 7, 8, 11, and 12). (C) Repolysis of the EGF receptor. Samples of dephosphorylated PM – i.e., B was repolysphorylated in the absence (lanes 13–16) or presence (lanes 17–20) of in vitro EGF. SDS-PAGE, alkali treatment, and radioautography (exposure for 16 h) was carried out as described in Materials and Methods. The band indicated as EGF-R corresponds to the EGF receptor as determined by immunoprecipitation with the monoclonal antibody IgG-L51, BH-6 (Fig. 6). Each lane represents 4.8 μg PM protein.
Ligand-Receptor Association in Endosomes

The cocktail of lectins, PEG, and γ-globulin along with Triton X-100 (Fig. 1) has indicated difficulty in optimizing conditions for binding EGF to its receptor in solubilized preparations. We have used past studies of Carpenter (9), as well as Yarden and Schlessinger (41), to bind to receptor in vitro during direct binding studies (15, 27). As discussed elsewhere (4), the small volume of endosomes (10⁻¹₇ liters) and the high concentration of intraendosomal ligand could act to promote ligand–receptor association and counteract the low pH-induced increase in $K_a$. The present observations on the intraendosomal association of EGF with its receptor are consistent with past observations in which the recovery of ligand–receptor complexes was assessed after direct binding to test membranes validated our protocol. At three doses of EGF injected (0.1, 1, and 10 μg EGF), receptor occupancy was evaluated by the above protocol. At peak times of internalization in the endosome (i.e., 15 min), the majority of internalized EGF was receptor bound for all doses of injected EGF. Greatest receptor occupancy (77%) was observed at the saturation dose as might be expected from the law of mass action.

The independent protocol of quantitative radioautography indicated that all of the EGF was membrane bound in isolated GE fractions at the 10-μg dose of EGF injected. This latter technique extended the biochemical determinations of Fig. 1; it demonstrated that the ligand–receptor complexes were associated with the bounding membrane of the liver endosome and not to any intraluminal vesicles. This is a noteworthy finding as it positions the receptor kinase to a site available for interaction with the cytosol.

The endosomal apparatus is acidic, although the acidity seems greater for late as opposed to early endosomes (6, 16). Nevertheless, even at pH 6, little EGF has been demonstrated to bind to receptor in vitro during direct binding studies (15, 27). As discussed elsewhere (4), the small volume of endosomes (10⁻¹⁷ liters) and the high concentration of intraendosomal ligand could act to promote ligand–receptor association and counteract the low pH-induced increase in $K_a$. The present observations on the intraendosomal association of EGF with its receptor are consistent with past observations in which the recovery of ligand–receptor complexes was assessed after direct binding to test membranes validated our protocol. At three doses of EGF injected (0.1, 1, and 10 μg EGF), receptor occupancy was evaluated by the above protocol. At peak times of internalization in the endosome (i.e., 15 min), the majority of internalized EGF was receptor bound for all doses of injected EGF. Greatest receptor occupancy (77%) was observed at the saturation dose as might be expected from the law of mass action.

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The endosomal apparatus is acidic, although the acidity seems greater for late as opposed to early endosomes (6, 16). Nevertheless, even at pH 6, little EGF has been demonstrated to bind to receptor in vitro during direct binding studies (15, 27). As discussed elsewhere (4), the small volume of endosomes (10⁻¹⁷ liters) and the high concentration of intraendosomal ligand could act to promote ligand–receptor association and counteract the low pH-induced increase in $K_a$. The present observations on the intraendosomal association of EGF with its receptor are consistent with past observations in which the recovery of ligand–receptor complexes was assessed after direct binding to test membranes validated our protocol. At three doses of EGF injected (0.1, 1, and 10 μg EGF), receptor occupancy was evaluated by the above protocol. At peak times of internalization in the endosome (i.e., 15 min), the majority of internalized EGF was receptor bound for all doses of injected EGF. Greatest receptor occupancy (77%) was observed at the saturation dose as might be expected from the law of mass action.

The independent protocol of quantitative radioautography indicated that all of the EGF was membrane bound in isolated GE fractions at the 10-μg dose of EGF injected. This latter technique extended the biochemical determinations of Fig. 1; it demonstrated that the ligand–receptor complexes were associated with the bounding membrane of the liver endosome and not to any intraluminal vesicles. This is a noteworthy finding as it positions the receptor kinase to a site available for interaction with the cytosol.
tions made by us and others indicating that internalized EGF is receptor bound within the endosomal apparatus. Thus, McKanna et al. (32) showed with ferritin-EGF in A431 cells a close association of ferritin with the bounding membrane of intracellular vesicles that we would now call endosomes but not with intraluminal vesicles of the temporally later multivesicular bodies. Lai et al. (28) using quantitative radiolaudography observed \(^{125}\)-EGF at the periphery of endosomes in placental syncytiotrophoblasts although radiolabel was found within the lumina of multivesicular bodies. By protein A-gold immunocytochemistry of Lowicryl-embedded A431 cells, Carpentier et al. (10) have also observed the majority of EGF associated with the intraluminal periphery of endosomes. Finally, Sorkin et al. (39) have concluded from studies using mild extraction protocols with the detergent Brij-58 that the majority of EGF internalized into endosomes of A431 cells is receptor bound. Taken together, therefore, the results from A431 cells, placenta, and liver parenchyma using a variety of biochemical and morphological methods all agree that the majority of EGF remains bound to its receptor in the bounding membrane of the endosome. This is relevant to a consideration of models for explaining EGF receptor traffic in liver and the relationship between internalization and transmembrane signaling of the EGF receptor kinase.

**EGF Receptor Autophosphorylation Activity**

An important consequence of the interaction of EGF with its receptor is the enhancement of receptor autophosphorylation activity. The relatively simple pattern of in vitro phosphorylated polypeptides of GE and PM fractions after incubation with \(\gamma^{32}\)-P[ATP] at 4°C and the ready identification of the 170,000-M. EGF receptor (20) which was confirmed further by immunoprecipitation studies with monoclonal antibody enabled the screening of EGF receptor autophosphorylation at several time points after the injection of various doses of EGF. For the GE fraction, from control (uninjected) rats, maximal autophosphorylation activity was observed in the presence of in vitro EGF provided the detergent Triton X-100 was present (20). (Similar results were observed in the absence of detergent except for latency in endosomes [20]). In the absence of in vitro EGF, a dose-dependent increase in autophosphorylation activity was observed. Thus, at maximal doses of injected EGF—i.e., 10 \(\mu\)g in the present study (Fig. 5) or 60 \(\mu\)g/100 g body weight in our past study (20)—little effect of in vitro EGF was observed on autophosphorylation activity. This increased basal (without EGF in vitro) autophosphorylation activity was most probably related to the increased proportion of receptor-bound EGF in the GE fraction at 15 min after the injection of 10 \(\mu\)g EGF (Fig. 1).

Therefore, ligand content, receptor content, the proportion of EGF bound to its receptor, and basal autophosphorylation activity are maximal in endosomes under conditions promoting receptor downregulation. These observations are all consistent with the hypothesis of Honegger et al. (19) that tyrosine kinase activity of the EGF receptor may control downregulation. In site-specific mutants of the EGF receptor where the ATP binding Lys 721 was substituted for Ala, downregulation was abolished although internalization was maintained. Although only a correlation between downregulation and elevated endosomal autophosphorylation activity has been demonstrated, it seems reasonable to speculate that some function of receptor kinase activity in the endosome such as interactions with a novel substrate may be linked causally to downregulation.

Additional studies are required to clarify the mechanism of downregulation and EGF receptor recycling. Thr 654 phosphorylation of the EGF receptor by protein kinase C results in receptor internalization and recycling but not downregulation (2). Recently, the E3 gene product of adenovirus has been shown to downregulate EGF receptor by a mechanism speculated to involve the formation of heterooligomers between the E3 gene product and the EGF receptor (8). As viral gene products may mimic normal cellular counterparts, then the dose dependence of downregulation we have observed may also reflect ligand-dependent receptor oligomerization with a yet to be described cellular counterpart to the E3 gene product.

PM fractions were also studied for autophosphorylation activity of the EGF receptor kinase. In confirmation of our previously published study (20) which used much higher doses of injected EGF (60 \(\mu\)g/100 g body weight), we observed here a near complete inhibition of autophosphorylation activity in the PM fraction at 15 min after injection. The dose-response studies (Fig. 5) showed, however, that this phenomenon was restricted to saturation doses (5 and 10 \(\mu\)g) of injected EGF. At lower doses, the diminution in autophosphorylation activity at 15 min approximated the drop in receptor content as assessed by direct binding (cf. Figs. 1 and 5 of the companion paper [29]).

The phenomenon was studied in greater detail. It was not related to rapidly turning over protein as dose-dependent inhibition of autophosphorylation activity at 15 min in PM was also observed in cycloheximide-treated rats. When this phenomenon was originally observed we suggested (20) that the inability to autophosphorylate in vitro may reflect a high level of autophosphorylation in vivo which would have occluded Tyr residues for further autophosphorylation in vitro. This was tested directly and found to be incorrect. The studies of Fig. 8 demonstrated that the EGF receptor in PM fractions isolated from control (uninjected) rats could be autophosphorylated in vitro, dephosphorylated, and then rephosphorylated. In the case of PM isolated at 15 min after the injection of a saturating dose of EGF, dephosphorylation did not result in the exposure of more available sites. Desensitization of the \(\beta\)-adrenergic receptor is regulated by a \(\beta\)-adrenergic receptor kinase that phosphorylates preferentially the ligand-occupied receptor as well as by phosphorylation from cAMP-dependent protein kinase C (3, 38). This model does not seem to apply, however, to the EGF receptor. Rather, desensitization may in this case involve (ligand) dose-dependent formation of a complex of receptor with a yet to be described desensitizing molecule within PM.

During the course of these studies, Kuppuswamy and Pike (26) demonstrated that 1 h after the incubation of 160 nM EGF to A431 cells the cells were desensitized for ligand-mediated internalization. We evaluated in liver parenchyma the internalization of ligand at the time of maximal desensitization of autophosphorylation. This study (Fig. 9) confirmed and extended the observation of Kuppuswamy and Pike (26). However, we found that the downregulated state per se (i.e., at 60 min) did not affect the ability to internalize low doses of EGF (Fig. 10). We therefore propose that desensitization of ligand internalization may be linked to the reduced state
of autophosphorylation activity in PM-located receptors but not simply to the downregulated state. The function of this transiently desensitized state may be to limit the extent of downregulation and would explain why, even during conditions of vastly excessive EGF (20), receptor loss from the PM is limited to 60–75% of the initial surface receptor content. Taken together with the recent demonstration of Glenny et al. (18) that ligand-mediated receptor internalization is blocked after the microinjection of anti-phosphotyrosine antibodies these studies suggest a link between the phosphorylated state of the EGF receptor and internalization.

In conclusion, our studies indicate that the EGF receptor content and autophosphorylation activities of the cell surface and internalized endosomally located receptor are regulated in a ligand-dependent fashion. The coincident inhibition of surface autophosphorylation activity and internalization as well as the enhancement of endosomal autophosphorylation activity coincident with downregulation suggests that EGF receptor traffic is regulated by ligand-mediated EGF receptor phosphorophorylation activity.

The authors thank Dr. F. Antohe for her participation in the radioautographic studies; Dr. B. Kopriwa for the preparation of the radioautographs; and Ms. M. Oeltzschner for excellent artwork. The excellent secretarial assistance of Ms. Prabha Ramamunhy and Ms. A. Innes is acknowledged.

Supported by grants from the National Cancer Institute of Canada (J. J. M. Bergeron) and the U. S. Public Health Service (B. I. Posner and J. J. M. Bergeron).

Received for publication 13 June 1989 and in revised form 28 August 1989.

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