Ligand-mediated Internalization, Recycling, and Downregulation of the Epidermal Growth Factor Receptor In Vivo

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Abstract. EGF receptor internalization, recycling, and downregulation were evaluated in liver parenchyma as a function of increasing doses of injected EGF. The effect of ligand occupancy in vivo on the kinetics and extent of internalization was studied with changes in the receptor content of isolated plasmalemma and endosome fractions evaluated by direct binding, Scatchard analysis, and Western blotting. For all doses of injected EGF, receptor was lost from the plasmalemma and accumulated in endosomes in a time- and dose-dependent fashion. However, at doses of injected EGF equivalent to ≤50% surface receptor occupancy (i.e., ≤1 μg/100 g body weight), receptor levels returned by 120 min to initial values. This return was resistant to cycloheximide and therefore did not represent newly synthesized receptor. Neither was the return due to replenishment by an intracellular pool of low-affinity receptors as such a pool could not be detected by Scatchard analysis or Western blotting. Therefore, receptor return was due to the recycling of previously internalized receptor.

At doses of injected EGF >50% receptor occupancy, net receptor loss—i.e., downregulation—was observed by evaluating the receptor content of total particulate fractions of liver homogenates. At the higher saturating doses of injected EGF (5 and 10 μg/100 g body weight), the majority of surface receptor content was lost by 15 min and remained low for at least an additional 105 min. As the kinetics of ligand clearance from the circulation and liver parenchyma were similar for all doses of EGF injected, then the ligand-mediated regulation of surface receptor content and downregulation were not a result of a prolonged temporal interaction of ligand with receptor. Rather, the phenomena must be a consequence of the absolute concentrations of EGF interacting with receptor at the cell surface and/or in endosomes.

SHORTLY after binding to target cells, EGF is internalized along with its receptor into components of the endosomal apparatus (10, 11, 14, 15, 19, 31, 34, 36). It is here that ligand and receptor are thought to be targeted for transport and destruction within lysosomes, leading ultimately to clearance of the ligand and downregulation (net loss) of receptor (9, 16, 23, 27, 35).

Liver parenchyma of male rats is enriched in EGF receptors (2, 14, 15, 19). Little is known of the relationship between surface recptor occupancy and the phenomenon of downregulation in an in vivo context. As well, the contribution of receptor recycling to ligand and receptor uptake is unclear (8, 14, 15, 22, 23, 27, 29). We have studied directly the in vivo interaction of EGF with its receptor by assessing the dose response of receptor content in plasmalemma (PM),[1] Golgi apparatus and endosome (GE), and total particulate (TP) fractions isolated from liver homogenates. We show that downregulation occurred only when >50% of surface receptors were occupied. At lower levels of surface receptor occupancy, receptor accumulated temporarily in endosomes but returned to the cell surface as a regulated recycling event.

1. Abbreviations used in this paper: GE, Golgi apparatus and endosome; PM, plasmalemma; TP, total particulate.

Materials and Methods

Materials

EGF was purchased from Collaborative Research (Waltham, MA). Carrier-free $Na^{125}I$ was purchased from DuPont Canada Inc. (Mississauga, Ontario, Canada). Reagents for SDS-PAGE were obtained from Bio-Rad Laboratories (Mississauga, Ontario, Canada). All other reagents and chemicals were of the highest purity available and were purchased as described previously (19).

Animals, Subcellular Fractionation, and Protein Determination

Male Sprague–Dawley rats (120 ± 5 g) were supplied by Charles River (St. Constant, Quebec, Canada). Rats were fasted for 16 h before use. PM was isolated by the method of Hubbard et al. (17) and modified as described previously (19). The GE fraction was also isolated as described previously (3) and used directly off the gradient without pelleting. GE fractions were recovered at a buoyant density of 1.09 g/cm^3. The fraction is a combined Golgi apparatus–endosome fraction free of PM contamination (19) and is designated the GE fraction for purposes of the work described herein. TP fractions were obtained by centrifugation of homogenates at 200,000 g_{av} for

30 min in 0.25 M sucrose with 4 mM immidazole, pH 7.5 (20). Protein was determined by the method of Bradford (6).

Ligand Iodination, Binding Assay, Scatchard Analysis, and Immunoblotting

EGF was iodinated using the chloramine T procedure as described previously (26). The specific activity of the ^{125}I-EGF was 180 ± 3.2 (SD) μCi/μg (n = 41). Direct binding studies were also carried out as described previously (25) with 25 mM Tris, pH 7.4, 10 mM $MgCl_2$, 0.1% BSA, 1,000 kallikrein inhibitor units/ml aprotinin, 40–50 μg/tube of PM or GE cell fraction protein, and 75,000 dpm ^{125}I-EGF in a final volume of 0.5 ml. Nonspecific binding was assessed by carrying out the incubations with excess unlabeled EGF (25 μg/ml). The binding assay for TP fractions (1 mg/tube cell fraction protein) was carried out in the presence or absence of 0.05% N-octyl-β-D-glucopyranoside (octylglucoside) at 4°C for 14 h with constant shaking and terminated by precipitation with polyethylene glycol and γ-globulin and filtration onto Whatman filters as described (12) or centrifugation at 2,000 g for 30 min at 4°C. Other detergents that were screened – i.e., Brij-35 (14, 15), Triton X-100, 3-([3-cholamidopropyl]dimethylammoniol)-1-propane-sulfonate, and Tween 20 – were considered unsuitable as these detergents at concentrations of 0.1–2% inhibited specific binding by 20–45% in TP fractions isolated from control (uninjected) rats. GE and TP fractions were also disrupted by freezing the fractions (−20°C) and thawing three times. Correction for endogenous EGF bound in vivo to the PM or GE fractions was by the method of Desbuquois et al. (13) in which endogenous EGF was removed with 0.2 N HCl containing 2% BSA and separated from membranes by recentrifugation and the supernatant was neutralized with 1 M Tris. The concentration of EGF in the neutralized supernatants was determined by radioreceptor assay and corrected values of EGF binding to membranes determined from the EGF content (Table I). The correction factors were taken as the ratio of ^{125}I-EGF bound to freshly prepared PM fractions in the absence of added EGF vs. ^{125}I-EGF bound in the presence of that EGF determined for the respective neutralized supernatants (see above).

The PM fraction revealed an average of 25.7 ± 3.6% (n = 16) specific binding/50 μg cell fraction protein, while GE fractions isolated from control rats showed an average of 6.9 ± 1.7% (n = 16) specific binding/50 μg cell fraction protein. Scatchard analyses were carried out on EGF inhibition dose-response data after correction for the presence of endogenous EGF according to the two-site model as described previously (28).

A peptide corresponding to residues 1,164–1,176 of the EGF receptor (i.e., KGSTRENREYLRV) was synthesized by Dr. N. Ling (The Salk Institute, San Diego, CA). The peptides were coupled to keyhole limpet hemocyanin, and polyclonal antibodies were raised after injection into rabbits. Immunoblotting was carried out on 50 μg protein of PM or GE fractions isolated at 0, 0.5, 5, and 15 min after the injection of 10 μg/100 g body weight EGF. The nitrocellulose paper containing the transferred protein was blocked with 5% BSA in a buffer consisting of 0.3 M NaCl, 0.5% Tween 20, 10 mM Tris-HCl, pH 7.4, for 30 min at room temperature and then blocked with 5% rat serum in the same buffer. The blocked nitrocellulose paper was treated with alkaline phosphatase at 37°C for 1 h and then incubated with the site-specific antibody (antiserum at 1:500 dilution) for 12 h at 4°C followed by ^{125}I-goat anti-rabbit IgG antibody for 1 h at room temperature. Quantitation of EGF receptor content was by densitometry of x-ray films exposed to the nitrocellulose paper for 18 h at −70°C. A linear response in signal was observed over a range of 5–100 μg of PM or GE protein subjected to immunoblotting by this protocol.

Electron Microscopy of PM and GE Fractions

For electron microscopy, subcellular fractions (50 μg cell fraction protein) were harvested straight from the density gradients and fixed immediately (see below) or incubated in 0.5 ml 25 mM Tris, 10 mM $MgCl_2$, pH 7.4, in the presence of 0.1% BSA at 4°C for 14 h with constant shaking as for the EGF binding assay described above (25). Membranes were fixed in 2.5% glutaraldehyde in 100 mM sodium cacodylate, pH 7.4, at 4°C. Samples containing 30 μg cell fraction protein were filtered under N_2 onto nitrocellulose filters (Millipore Continental Water Systems, Bedford, MA) using a filtration apparatus as described previously (1). Filters were postfixed in 2% OsO_4 in 100 mM cacodylate buffer, pH 7.4, and stained en bloc with 1% tannic acid (32) and 8% uranyl acetate in 100 mM maleic buffer, pH 5 (18), and processed for routine electron microscopy.

Inhibition of Protein Synthesis by Cycloheximide

The efficacy of cycloheximide inhibition of protein synthesis was evaluated. At 60 min after the intraperitoneal injection of cycloheximide (0.2 mg/100 g body weight) [^{3}H]L-leucine (2.5 μCi/120 g body weight, 5 Ci/mmol sp act) was injected into the portal vein of rats injected with either cycloheximide or PBS. TCA precipitation of liver homogenates prepared from rats killed at various times (1–8 h) after the injection of [^{3}H]leucine showed >95% inhibition of incorporation compared with liver homogenates from animals not injected with cycloheximide. For the experiments described in Results, a cycloheximide dose of 0.2 mg/100 g body weight was injected 120 min before the injection of EGF.

Clearance and Hepatic Uptake of ^{125}I-EGF

^{125}I-EGF (0.1, 1, and 10 μg/100 g body weight) was injected into the hepatic portal vein of anesthetized fasted rats. At various times after ^{125}I-EGF injection, plasma was collected from the aorta and centrifuged at 4°C at 1,500 g for 15 min. Livers were quickly placed in ice-cold 0.25 M sucrose containing 4 mM immidazole, pH 7.5, to yield a 20% homogenate. Plasma samples and liver homogenates were precipitated with ice-cold 10% TCA for 30 min, and TCA-precipitable radioactivity was determined.

Results

Dose Response and Kinetics of Translocation of Binding Sites from the Cell Surface to Endosomes

The kinetics and dose response of receptor internalization

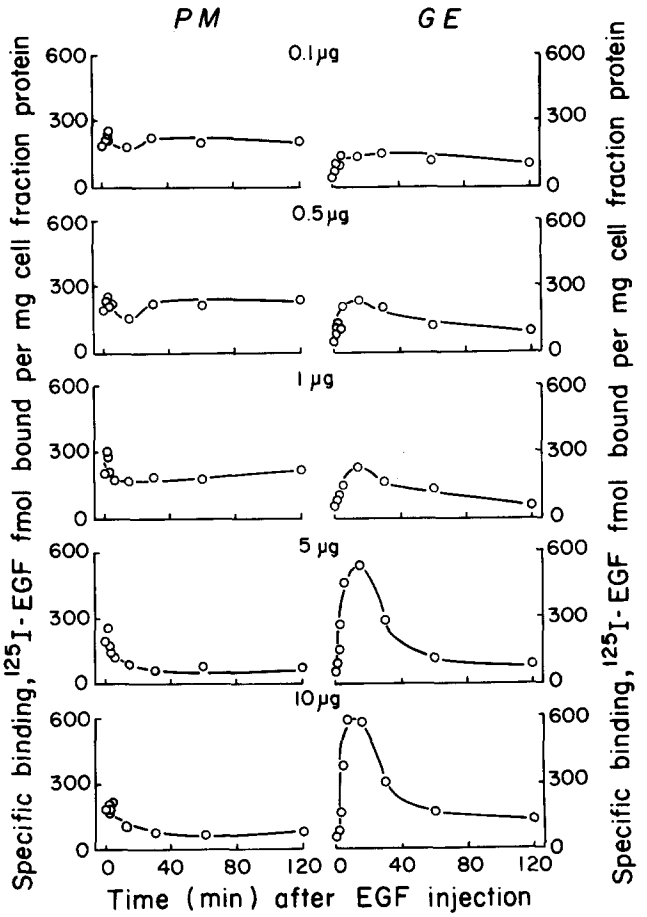


Figure 1. Specific binding of 10^{-10} M ^{125}I-EGF to PM and GE fractions isolated at various times after the injection of 0–10 μg EGF/100 g body weight as described in Materials and Methods. Direct binding with 10^{-10} M ^{125}I-EGF was carried out on freshly prepared fractions and corrected for the presence of endogenous EGF as described in Materials and Methods and Tables I and II and expressed as femtomoles of EGF specifically bound per milligram of cell fraction protein.

Table I. EGF Content of Subcellular Fractions

Subcellular fraction	Dose of EGF injected	EGF content at various times (min) after EGF injection							
		0.5	1	2.5	5	15	30	60	120
	μg/100 g body weight	*pmol/mg cell fraction protein*							
PM	0.1	0.51	0.51	0.51	0.48	0.46	0.51	0.15	0.15
	1	1.75	1.52	1.47	1.34	1.15	1.15	0.49	0.28
	10	5.30	4.64	4.64	4.30	4.14	2.67	0.99	0.71
GE	0.1	0.50	0.60	0.81	1.09	1.16	1.13	0.60	0.50
	1	1.59	1.67	3.76	4.82	5.84	4.13	2.98	0.41
	10	2.30	4.92	20.15	29.77	23.39	16.00	7.09	4.01
TP	0.1	0.15	0.17	0.13	0.07	0.08	0.03	0.08	0.08
	1	0.31	0.30	0.30	0.30	0.30	0.22	0.17	0.10
	10	0.77	0.77	1.00	1.25	1.66	1.17	1.10	0.34

Subcellular fractions were prepared at the indicated times after the injection of 0.1, 1, and 10 μg of EGF/100 g body weight, and EGF content was determined by radioreceptor assay as described in Materials and Methods. Data are averages from two or more separate experiments.

and reappearance on the cell surface were evaluated for the binding domain of the EGF receptor. As before (19), subcellular fractions corresponding to the hepatic PM and a combined GE fraction were isolated from liver homogenates at various times after the portal vein injection of EGF. No difference was observed whether direct binding studies were carried out on freshly prepared fractions or after freezing and thawing of the fractions three times. After 14 h of incubation in the hypotonic buffer used for the binding assays, electron microscopy revealed that GE fractions were disrupted, thereby accounting for the lack of latency in binding to this fraction (not shown). Direct binding studies on the fractions showed the temporal loss of binding sites from PM that coincided with entry into GE fractions (Fig. 1). No lag (i.e., ≤30 s) was observed in the accumulation of receptor into the GE fraction. The maximal extent of receptor loss from the PM occurred by 15 min and appeared dose dependent as did the extent of accumulation of receptor into the GE fraction at peak times of internalization, which were also at 15 min.

Unexpectedly, the receptor content of PM fractions after the injection of 0.1, 0.5, and 1 μg of EGF returned to near initial values by 30 min after the injection of the 0.1- and 0.5-μg doses and by 120 min after the 1-μg dose. For the 5- and 10-μg doses, the receptor content of the PM fractions remained low from 15 to 120 min after EGF injection.

To derive the data of Fig. 1, direct binding was corrected for the presence of endogenous ligand in the fractions as a consequence of the doses of EGF injected. EGF content in the cell fractions was evaluated by radioreceptor assay (Table I) and as expected was dose and time dependent. GE fractions accumulated considerable quantities of EGF compared with PM fractions. Subsequent correction factors (Table II) were calculated as originally described by Desbuquois et al. (13).

Scatchard Analysis and Immunoblotting

To verify that the specific binding assays were representative of changes in receptor number and not affinity, Scatchard analyses were carried out at selected doses and time intervals. The results (Fig. 2) revealed curvilinear plots that could be resolved into a minority (~18%) of high-affinity and a majority of low-affinity sites (Table III). One caveat concerned the GE fraction isolated from livers of rats injected 15 min previously with 10 μg EGF. For these conditions, the high concentration of internalized ligand (Table I) vitiated the calculations of receptor content and affinity constants by the LIGAND program (28), which was used as the quantity

Table II. Correction Factor for Direct Binding of ^{125}I-EGF to Subcellular Fraction

Subcellular fraction	Dose of EGF	Correction factor at various times (min) after EGF injection							
		0.5	1	2.5	5	15	30	60	120
	μg/100 g body weight								
PM	0.1	1.21	1.20	1.20	1.20	1.19	1.20	1.06	1.06
	1	1.44	1.41	1.39	1.39	1.35	1.35	1.20	1.13
	10	1.87	1.82	1.82	1.78	1.78	1.56	1.32	1.26
GE	0.1	1.19	1.23	1.28	1.33	1.35	1.35	1.23	1.19
	1	1.41	1.43	1.72	1.82	2.00	1.75	1.61	1.19
	10	1.47	1.85	3.70	5.00	4.17	3.23	2.13	1.72
TP	0.1	1.06	1.06	1.05	1.01	1.03	1.00	1.01	1.01
	1	1.14	1.13	1.13	1.13	1.13	1.10	1.06	1.03
	10	1.28	1.28	1.32	1.40	1.44	1.36	1.32	1.16

Correction factors were calculated as the ratio of ^{125}I-EGF bound to control (uninjected) PM fractions (mean of 10 preparations) in the absence of cold EGF and ^{125}I-EGF bound in the presence of that EGF content determined from Table I as described in Materials and Methods.

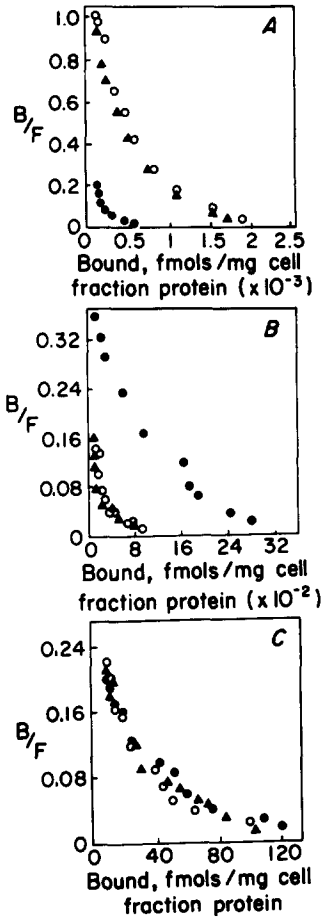


Figure 2. Scatchard analysis of ^{125}I-EGF binding to (*A*) PM, (*B*) GE, and (*C*) TP fractions. PM, GE, and TP fractions were prepared from control (uninjected) (○) and at 15 (▲) and 120 min (●) after the injection of 1 μg EGF/100 g body weight.

of endogenous internalized EGF even by 2.5 min after EGF injection was saturating. Consequently, immunoblotting was carried out with a site-specific antibody to the cytosolic domain of the EGF receptor as described in Materials and Methods. A time-dependent decrease in receptor content was observed for PM, with a marked progressive increase in receptor content observed in GE fractions isolated from liver homogenates at 0–15 min after ligand injection. Quantitation by densitometry (Fig. 3, *bottom*) confirmed the temporal differences in receptor content for the two fractions.

Dose of EGF Injected and In Vivo Receptor Occupancy

An in vivo inhibition dose-response study was carried out for PM and GE fractions isolated at 15 min after the injection of tracer doses of ^{125}I-EGF and increasing doses of unlabeled EGF (Fig. 4). The extent of receptor occupancy was calculated by converting the data to femtomoles EGF bound or internalized per milligram cell fraction protein. At doses of 5–10 μg EGF injected, maximal surface receptor occupancy was observed. From the in vivo displacement curves at 0.1, 1, and 10 μg EGF injected, estimates of receptor occupancy were 7%, 50%, and saturation, respectively.

Receptor Recycling and Downregulation

The observations of Figs. 1 and 2 were pursued in greater detail. Notably, the unexpected return by 120 min to basal (zero time) levels of receptor content in PM fractions after the injection of 0.1, 0.5, and 1 μg of EGF/100 g body weight was considered as a possible receptor recycling event. The lack of receptor return at the 5- and 10-μg doses was considered as a possible reflection of ligand-mediated downregulation.

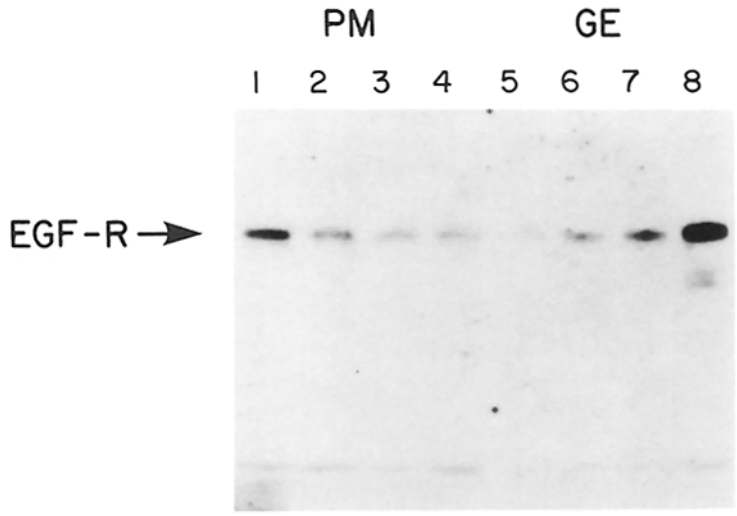
Replicate studies were carried out on control (uninjected) rats and also at 15 and 120 min after the injection of 0.1, 1, and 10 μg EGF (Fig. 5) confirming the observations of Figs. 1 and 2. To assess the contribution of newly synthesized receptor to the dose-regulated return of receptor in PM at 120 min, identical experiments were carried out in cycloheximide-treated rats (Fig. 5) in which protein synthesis was inhibited by >95%. Although receptor loss was more marked at 15 min after ligand injection, receptor content in the PM fraction returned to near initial levels for the 0.1-μg dose of injected EGF and to 80% of initial levels at the 1-μg dose. At the 10-μg dose, the receptor content of PM at 120 min was identical to that at 15 min after ligand injection.

Although the return by 120 min to basal receptor levels in cycloheximide-treated rats was consistent with recycling, an alternative hypothesis has been proposed by Dunn et al. (15)

Table III. Scatchard Analysis of ^{125}I-EGF Binding to Subcellular Fractions

Subcellular fraction	EGF injected	Time after EGF injection	Binding parameters			
			K_1	N_1	K_2	N_2
	μg/100 g body weight	*min*	*×10⁹ M⁻¹*	*fmol/mg*	*×10⁸ M⁻¹*	*fmol/mg*
PM	0	0	8.2	512	2.8	2,125
	1	15	7.8	121	2.1	875
	1	120	7.2	502	2.4	2,050
GE	0	0	7.4	162	4.1	927
	1	15	15.1	614	3.7	2,702
	1	120	7.2	176	4.3	989
TP	0	0	8.1	38	2.9	146
	1	15	8.1	32	3.3	138
	1	120	7.2	34	2.7	140

Binding parameters were determined from Scatchard analyses that were fitted on the basis of a two-site model (28) to obtain receptor number (N_1, high affinity receptor; N_2, low affinity receptor) and affinity constants (K_1, high affinity constant; K_2, low affinity constant). Binding was carried out at 4°C for 14 h as described in Materials and Methods. In the TP fractions, binding was carried out in the presence of octylglucoside.



Time after EGF injection (min)	0	0.5	5	15	0	0.5	5	15
Densitometry (arbitrary units)	1.08	0.42	0.27	0.25	0.15	0.29	0.71	2.27

Figure 3. Immunoblotting of the EGF receptor in PM and GE fractions isolated from control (uninjected) rats and at 0.5, 5, and 15 min after the injection of 10 μg EGF/100 g body weight. Immunoblotting was carried out on 50 μg of cell fraction protein of the PM (lanes *1–4*) and GE fractions (lanes *5–8*) as described in Materials and Methods. At the bottom of each lane is indicated the amount of EGF receptor as determined by densitometry in arbitrary units.

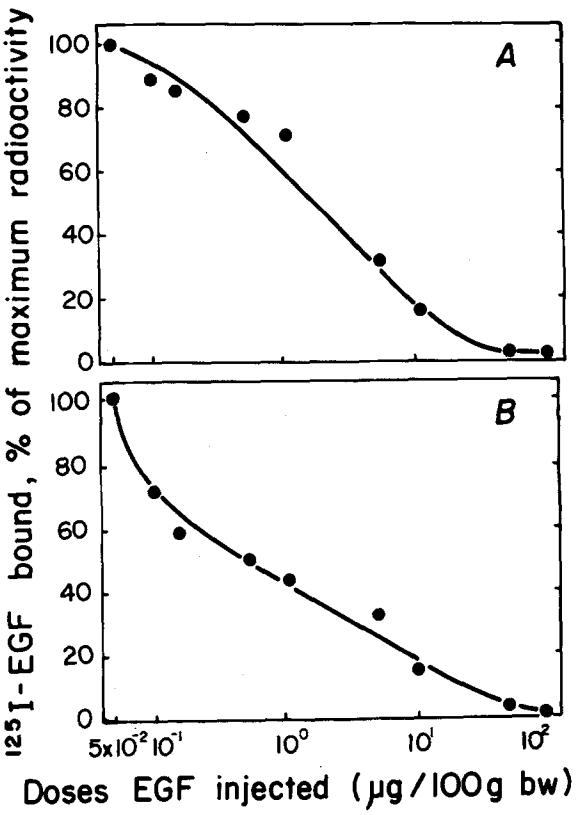


Figure 4. In vivo inhibition dose response of ^{125}I-EGF binding to PM and GE fractions. Livers were rapidly excised 15 min after the injection of 22 × 10^6 dpm ^{125}I-EGF (210 μCi/μg sp act) that was coinjected with increasing amounts of unlabeled EGF (0.05–100 μg/100 g body weight). PM (*A*) and GE fractions (*B*) were prepared, and the amount of radiolabeled EGF bound to the fractions was determined by gamma counting. Calculation of femtomoles of hormone bound showed that saturation was achieved at 5–10 μg EGF injected.

in which a large pool of low affinity intracellular receptors were suggested to replenish the surface EGF receptor content after ligand-mediated internalization. Accordingly, we attempted to evaluate the existence of such a pool.

TP fractions were isolated from livers of male rats at various times after the injection of increasing doses of EGF (Fig. 6). When direct binding studies were carried out, the data, after correction for the endogenous content of EGF, showed a rapid ($t_{½}$ < 1 min) loss of binding sites at the 1- and 10-μg doses. The extent of receptor loss was maximal at 15 min and was dose dependent. That this loss was due to entry of receptor into a cryptic compartment was demonstrated when the binding studies were carried out on fractions treated with the detergent octylglucoside (Fig. 6, *solid symbols*) or on the TP fraction frozen and thawed three times (not shown). Under these conditions, no loss of binding sites was observed at doses of 0.1 or 1 μg EGF injected. Only at the 10-μg dose was a slower exponential loss of binding sites observed ($t_{½}$ ~ 2 h).

Scatchard analysis of EGF binding to the TP fraction (Fig. 2 *C*) showed identical curvilinear Scatchard plots to those observed for PM and GE fractions. By the two-site model, similar affinities of the high and low affinity receptors to those in PM and GE fractions were found (Table III). Only the receptor concentration was lower than that for the PM and GE fractions. The Scatchard analyses on TP fractions also permitted an estimate of receptor yields in the PM and GE fractions (Table IV).

The suggestion (15) of a pool of cryptic low affinity receptors of K_d ~ 200 nM was not supported by the studies of Fig. 2. Direct binding studies (Figs. 1 and 6) were consistent with the immunoblot data carried out at the 10-μg dose (Fig. 3) and were considered reflective of receptor content. Consequently, the effect of injected EGF on receptor content of liver parenchyma was assessed by direct binding (Fig. 7). Only at 5- and 10-μg doses of injected EGF was net receptor

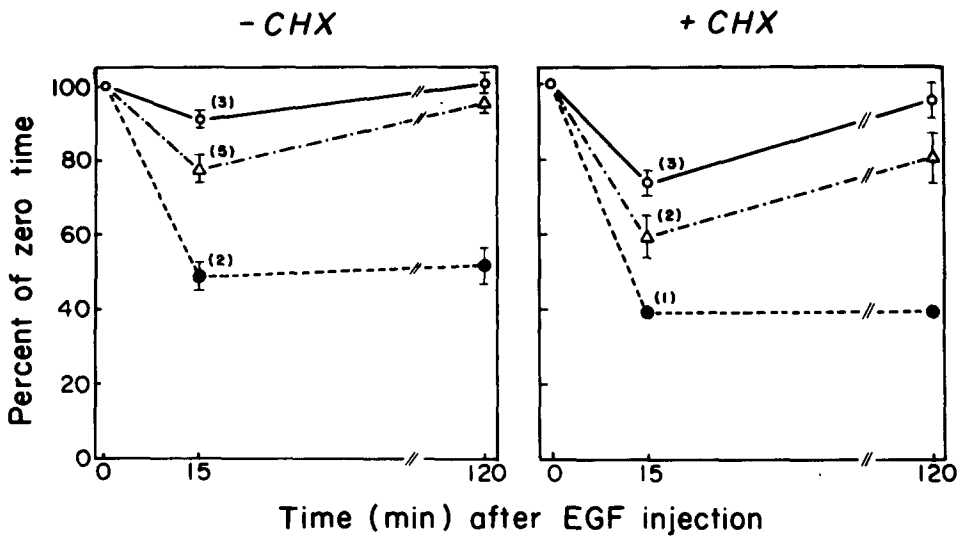


Figure 5. Reappearance by 120 min of initial EGF receptor content of PM at low (0.1 and 1 μg) but not high (10 μg) doses of EGF injected. Specific binding was carried out on PM fractions isolated at 0 (without EGF injection), 15, and 120 min from rats injected with 0.1 (○), 1 (△), and 10 μg EGF (●) from control (without cycloheximide) (−*CHX*) or cycloheximide-treated rats and corrected for the endogenous content of EGF as described in Materials and Methods. Parentheses refer to the number of experiments for each time point (mean ± SD for $n \geq 3$ or mean ± half of the variation for $n = 2$). All binding assays for each experiment were carried out in triplicate. Slightly lower receptor levels were observed in PM fractions isolated at 15 and 120 min after ligand injection into cycloheximide-treated rats as compared with control rats. The difference presumably represents the pool of newly synthesized receptors.

loss (i.e., downregulation) observed at 120 min after ligand injection. Immunoblotting on TP fractions was not possible due to the lack of sensitivity of the procedure in the crude fraction.

Ligand Clearance

The binding studies carried out on the TP fractions (Fig. 6) and PM and GE fractions (Fig. 1) were consistent. Taken together, the observations indicated that receptor was internalized in a ligand-mediated fashion. At low subsaturating doses of injected ligand (<1 μg), receptors returned to initial levels on the cell surface but were lost at higher ligand doses. The contribution of ligand availability in vivo to recycling and downregulation was next addressed. ^{125}I-EGF was injected into the portal vein at doses of 0.1, 1, and 10 μg/100 g body weight, and the content of TCA-precipitable radiolabel was evaluated in plasma samples and liver homogenates (Fig. 8). Rapid loss from the circulation (Fig. 8) of TCA-precipitable ^{125}I-EGF was found for all doses of EGF injected. Curiously, between 30 and 60 min after injection, TCA-precipitable ^{125}I-EGF reappeared in the circulation after the 0.1- and 1-μg doses of injected EGF (Fig. 8, *arrows*) but not the 10-μg dose. The clearance of ligand from liver was more prolonged than from plasma but with similar overall rates observed for the different doses of injected EGF such that by 90 min after injection, 78, 85, and 80% of maximal levels of radiolabel were lost from liver after the injection of 0.1, 1, and 10 μg EGF, respectively.

Discussion

These studies have defined the in vivo dose response of receptor internalization, recycling, and downregulation of the EGF receptor in liver parenchyma. The binding of labeled EGF to receptors in defined subcellular fractions isolated from liver homogenates was considered an accurate reflection of changes in receptor content occurring at the cell surface and in endosomes. We have previously used identical protocols for separating PM from GE fractions to study EGF receptor kinase activity therein (19). Based on SDS-PAGE and electron microscopy of random views of the fractions, we confirmed that the fractions were not cross-contaminated (data not shown). Hubbard et al. (17) have characterized the PM fraction extensively. The GE fraction consists of a mixture of Golgi apparatus and endosomes. The fraction has been previously characterized enzymatically and morphologically (3, 4, 33) as well as by electron microscopic radioautography for the components containing internalized ^{125}I-EGF (5, 19).

That the direct binding studies reasonably reflected receptor content was a consideration of the Scatchard analyses of Fig. 2 and the immunoblot data of Fig. 3. No difference in the affinity constants of the EGF receptor was noted in PM and GE fractions. An identical conclusion was made by Desbuquois et al. (13) and Khan et al. (21) who studied the insulin receptor in PM and GE fractions by similar methods. Hence, the direct binding studies carried out at 10^{-10} M ^{125}I-EGF were not vitiated by changes in affinity that might have occurred to the receptor during internalization. The immunoblots were carried out at a saturating dose (10 μg EGF) of injected ligand. These studies showed that the receptor content changed in PM and GE fractions similar to that observed by direct binding. Hence, direct binding after consideration and correction for the content of endogenous ligand was a valid estimate of the relative receptor content in the PM and GE fractions.

The EGF receptor is considered to be an example of a receptor that is degraded shortly after ligand-mediated internalization (14, 15, 22, 23, 27, 35). It was therefore unexpected that after internalization, the receptor content in PM fractions returned to starting levels and that the extent of this return was related approximately inversely to the dose of ligand injected. Experiments carried out on cycloheximide-treated rats ruled out the possibility that the majority of receptors returning to the cell surface were newly synthesized. Receptor recycling was therefore considered as an ex-

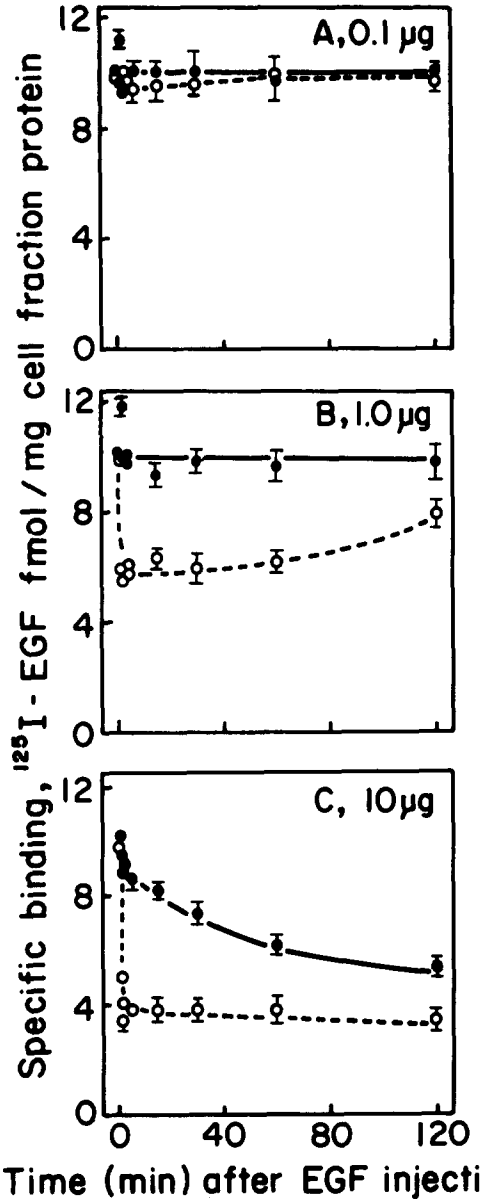


Figure 6. ^{125}I-EGF specific binding to TP fractions at various times after the injection of (*A*) 0.1, (*B*) 1, and (*C*) 10 μg EGF/100 g body weight. Rats were killed at the indicated times after the injections of EGF, the TP fraction was isolated from liver homogenates, and specific binding was carried out with 10^{-10} M ^{125}I-EGF in the presence (•) or absence (○) of 0.05% octylglucoside. Specific binding was evaluated and corrected for the presence of endogenous EGF as described in Materials and Methods and Tables I and II and expressed as femtomoles EGF specifically bound per milligram cell fraction protein. Results are the mean of three separate experiments ± SD.

planation of the results. (Murthy et al. [29] have also reported the recycling of EGF receptors in murine 3T3 and human WI-38 cells; however, observations based on direct binding were not evaluated for the contribution of newly synthesized EGF receptor.)

To account for the continued endocytosis of EGF in perfused livers treated with cycloheximide, Dunn et al. (15) postulated the existence of a large pool of cryptic intracellular low affinity EGF receptors that would replenish surface-located EGF receptors after internalization. According to this model, receptor recycling is not necessary to account for their findings. Our attempts to identify, by direct binding, the existence of such a pool were without success. TP fractions revealed receptors of the same affinities as PM and GE fractions even after treatment with detergent to expose any latent binding sites. (Latency for binding was observed in TP fractions but not GE fractions after 14 h of incubation in hypotonic buffer. This may be related to the difference in protein concentration of the respective fractions in the binding assay; protein concentrations of TP and GE fractions were 2 and 0.1 mg/ml, respectively. Electron microscopy showed that GE fractions after 14 h of incubation were disrupted, permitting access of the ^{125}I-EGF to intraluminal binding sites within endosomes.) Furthermore, quantitative immunoblot-

Table IV. Yield and Receptor Content of Subcellular Fractions Isolated from Liver Homogenates of Control (Uninjected) Rats

Subcellular fraction	Protein yield of fraction*	Receptor content‡
	mg/g liver (n)	*No./g liver × 10^{-11}*
TP	123.7 ± 1.9 (44)	137
PM	1.19 ± 0.2 (32)	19
GE	0.59 ± 0.2 (42)	4

* No significant difference was found in the yield of fractions isolated from uninjected or EGF-injected rats. Consequently, the values represent the mean of all experiments ± SD. The number of separate experiments is indicated in parentheses.
‡ Receptor content was calculated from Table III and represents the sum of low and high affinity receptors.

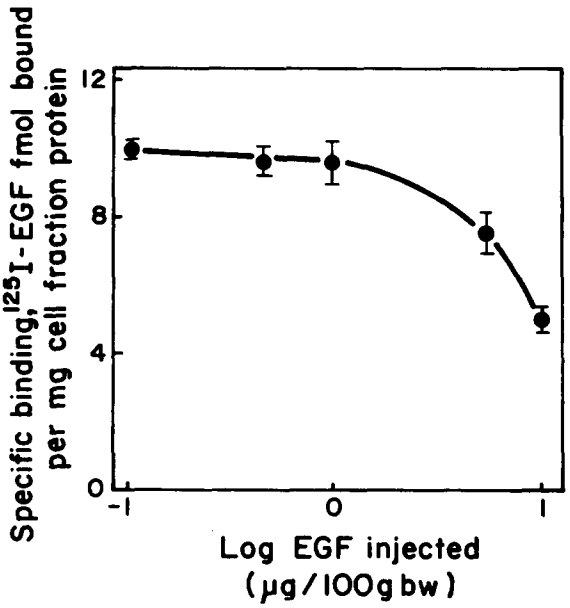


Figure 7. Specific binding of 10^{-10} M ^{125}I-EGF in the presence of octylglucoside (0.05%) to rat liver TP fractions isolated at 120 min after the injection of 0.1, 0.5, 1, 5, and 10 μg EGF/100 g body weight. Data were from three separate experiments for each dose of EGF injected (mean ± SD).

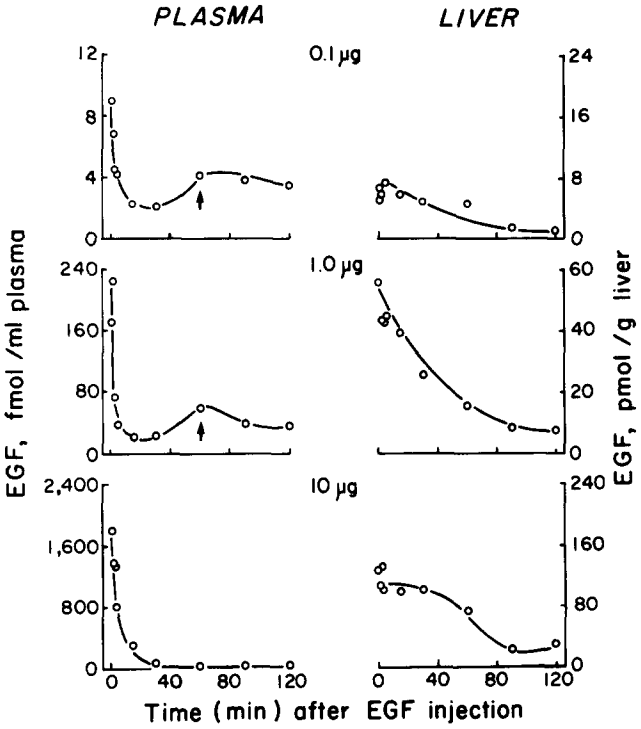


Figure 8. Clearance of injected EGF from the circulation (*left*) or liver parenchyma (*right*). 0.1, 1, and 10 μg ^{125}EGF (180 μCi/μg sp act)/100 g body weight was injected into the hepatic portal vein. At various times thereafter, plasma was collected from the aorta. Plasma samples and liver homogenates were precipitated with ice-cold 10% TCA for 30 min, and TCA-precipitable radioactivity was determined. The arrows point to increases in TCA-precipitable radiolabel in plasma occurring between 30 and 60 min after injection. Despite the different doses of EGF administered, the kinetics of clearance for each dose from the circulation or liver parenchyma are remarkably similar.

ting with the site-specific antibody to the cytosolic domain of the EGF receptor failed to detect a high concentration of receptors in the GE fraction isolated from control (uninjected) rats (Fig. 3, lane *5*). The GE fraction was recovered at a buoyant density of 1.09 g/c^3 on sucrose gradients—i.e., within the density range (1.08–1.12 g/cm^3) of the compartment reported (15) to harbor the low affinity EGF receptors. We therefore could not confirm the presence of an extensive cryptic pool of low affinity receptors and conclude that previously internalized receptor recycled to the cell surface when surface receptor occupancy was ≤50%. Also noteworthy was the finding that a proportion of internalized ligand also reappeared in the circulation between 30 and 60 min after the injection of 0.1 and 1 μg EGF but not 10 μg EGF (Fig. 8, *arrows*), suggesting that a portion of the reappearing receptors were ferrying previously internalized ligand and/or partially processed ligand (24, 31, 38).

From the receptor content and yield of TP fractions (Table IV) the number of EGF receptors per hepatocyte may be calculated. Weibel et al. (37) have estimated 1.58 × 10^8 hepatocytes for 1 g liver. Since all membrane-bound EGF receptors would be represented in the TP fraction we then calculate 87 × 10^3 EGF receptors per hepatocyte. This number is less than that originally calculated by Dunn et al. (14) from Scatchard analyses of ^{125}I-EGF binding to liver homogenates. The yield of receptors recovered in PM and GE fractions could also be estimated from Table III, with 13.9% of total receptors recovered in PM fractions and 2.9% in GE fractions isolated from control (uninjected) rats, and indicates the limitations (low yield) of the preparative subcellular fractionation protocols.

The studies with the TP fraction also defined the dose response of downregulation in liver parenchyma. After saturating doses of injected ligand, net receptor loss was observed in TP fractions concomitant with a lack of receptor replenishment in PM fractions. These studies do not rule out recycling in downregulation as only net changes in receptor pools and not the flux of individual receptors was measured in our studies. Nevertheless, the changes in receptor content of the PM were ligand mediated. The molecular signals for receptor internalization are as yet poorly defined although ligand-mediated receptor oligomerization and trapping of EGF and low density lipoprotein receptor cytosolic domains by adaptins in clathrin-coated pits have been proposed as has receptor phosphorylation (7, 30). After internalization, however, our studies indicate that a choice is made, presumably within the endosome. Either the receptors return to maintain the initial surface receptor level or they are transported to a degradative compartment, presumably the lysosome. The choice must be some consequence of the concentration of internalized ligand and receptor but not the time with which ligand remains available to receptor as clearance was similar from the circulation or liver parenchyma for all doses of injected EGF.

We gratefully acknowledge the synthesis of peptides by Dr. N. Ling of the Salk Institute. The authors also thank Ms. M. Oeltzschner for her art work and for the secretarial assistance of Ms. Prabha Ramamurthy.

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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