Receptor-mediated Endocytosis of Epidermal Growth Factor by Rat Hepatocytes: Receptor Pathway

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Abstract. Substantial amounts of epidermal growth factor (EGF) are cleared from the circulation by hepatocytes via receptor-mediated endocytosis and subsequently degraded within lysosomes. We have used a combined biochemical and morphological approach to examine the fate of the receptor after exposure to EGF. Polyclonal antibodies were prepared against the purified receptor and their specificity established by immunoprecipitation and immunoblotting techniques. The EGF receptor was then localized by immunofluorescence and immunoperoxidase techniques and quantified on immunoblots. In untreated livers, EGF receptor was restricted to the sinusoidal and lateral surfaces of hepatocytes. 2-4 min after exposure of cells to EGF, the receptor was found in small vesicles (i.e., coated vesicles) as well as larger vesicles and tubules at the cell periphery. By 15 min the receptor was found in multivesicular endosomes located near bile canaliculi. Exposure of hepatocytes to EGF also resulted in a rapid loss of receptor protein from total liver homogenates and a decrease in its half-life from 8.7 h in control livers to 2.5 h. This EGF-induced loss of receptors was not observed when lysosomal proteinases were inhibited by leupeptin or when endosome/lysosome fusion was prevented by low temperature (16°C). In the presence of leupeptin, receptor could be detected in structures identified as lysosomes using acid-phosphatase cytochemistry. All these results suggested rapid internalization of EGF receptors in response to ligand and degradation within lysosomes. However, four times more ligand was degraded at 8 h than the number of high-affinity (Kₜ of 8-15 nM) EGF-binding sites lost, suggesting either (a) high-affinity receptors were recycled, and/or (b) more than 300,000 receptors were available for EGF uptake. In the presence of leupeptin, receptor could be detected in structures identified as lysosomes using acid-phosphatase cytochemistry. All these results suggested rapid internalization of EGF receptors in response to ligand and degradation within lysosomes. However, four times more ligand was degraded at 8 h than the number of high-affinity (Kₜ of 8-15 nM) EGF-binding sites lost, suggesting either (a) high-affinity receptors were recycled, and/or (b) more than 300,000 receptors were available for EGF uptake. We identified and characterized a latent pool of ~300,000 low-affinity receptors (Kₜ ~ 200 nM) that could be separated on sucrose gradients from the plasma membrane pool of ~300,000 high-affinity receptors (Kₜ of 8-15 nM). Despite the differences in their binding affinities, the high- and low-affinity receptors appeared to be structurally identical and were both EGF-dependent protein kinases. In addition, the dynamics of the low-affinity receptors were consistent with a functional role in EGF uptake and delivery to lysosomes.

Receptor-mediated endocytosis is well-recognized as a general mechanism used by many cells for uptake of biologically important molecules (reviewed in references 7 and 51). The fates of these ligands, once internalized, vary from degradation to intracellular storage to transport across the cell and subsequent release. The receptors also have diverse fates, some being re-utilized, others degraded, and a few released with their ligand. The principle cell in the liver, the parenchymal cell or hepatocyte, possesses many specific membrane receptors that mediate the internalization of different molecules from the circulation. The fates of these ligands and their receptors encompass most currently established fates in receptor-mediated endocytosis. Therefore, this cell type is particularly well-suited for a comparative study of multiple ligand–receptor systems.

We have been studying the endocytosis of asialoglycoproteins (ASGPs), epidermal growth factor (EGF), and polymeric immunoglobulin A by rat hepatocytes (18-20, 28, 29, 56). These molecules are internalized via receptor-mediated processes occurring at the sinusoidal surface of hepatocytes. Two of the ligands (ASGP and EGF) are delivered to lysosomes and degraded (2, 17, 20), but the third, polymeric immunoglobulin A, is transported across the cell and excreted into the bile (46). While the receptors for ASGPs are re-utilized (50), membrane secretory component, the receptor for polymeric immunoglobulin A, is apparently transported and a 90-kD fragment secreted into the bile along with the ligand (Hoppe, C. A. and A. L. Hubbard, unpublished results). Evidence from studies of cultured cells indicates that the EGF receptor is delivered to lysosomes and degraded together with the ligand (6, 14, 34, 52). However, we recently reported that more EGF was internalized and degraded by hepatocytes than...
functional receptors present, even when protein synthesis was inhibited (20), suggesting that EGF receptors were re-utilized. An alternative possibility was that additional or cryptic receptors were recruited for continued ligand internalization.

In this report, we have used anti-receptor antibodies to investigate the dynamics of the EGF receptor in more detail. We have found that EGF induces a redistribution of receptors from the cell surface through all endosomal compartments to lysosomes. Furthermore, the amount of total receptor protein decreases upon exposure to ligand, indicating that it is degraded. Finally, we have identified a large pool of low-affinity receptors that can be separated from the high-affinity receptors on sucrose gradients of subcellular fractions. The size (~50% of total receptor protein) and dynamics of this low-affinity pool are consistent with its functioning in the continued endocytosis of EGF.

Materials and Methods

Materials

CD Sprague-Dawley rats were purchased from Charles River Breeding Laboratories (Wilmington, MA). EGF was prepared from mouse submaxillary glands (Pel-Freeze Biologicals, Rogers, AR) as described by Savage and Cohen (44) and iodinated to specific radioactivities of 2.6 × 10^6 cpm/μg (25). Affi-Gel 10 was obtained from Pharmacia Inc. (Piscataway, NJ); Sepharose 4B from Sigma Chemical Co. (St. Louis, MO); Trasylol from Mobay Chemical Corp. (Pittsburgh, PA); ascorbate, leupeptin, and peptastatin were from Peninsula Laboratories, Inc. (Belmont, CA); complete Freund’s adjuvant and incomplete Freund’s adjuvant were from Difco Laboratories Inc. (Detroit, MI); rhodanomated goat anti-rabbit IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD); and horseradish peroxidase-conjugated sheep anti-rabbit Fab was from Pasteur Productions (Marnes La Coquette, France). Protein A (Pharmacia Inc.) was iodinated using the chloramine T method (25) with resulting specific radioactivities of 3.5 × 10^6 cpm/μg. Adenosine-5’-[32p]triphosphate (10-25 Ci/mmol) was from ICN Pharmaceuticals, Inc. (Irvine, CA). All other reagents and compounds were of the highest purity available and were purchased from commercial sources or from sources reported in earlier publications (20, 30, 42).

Purification of EGF Receptor and Preparation of Antibodies to the Receptor

EGF-Affi-Gel 10 (0.7–1.0 mg of packed bed volume) was prepared and EGF receptor purified from rat liver by affinity chromatography as described by Cohen et al. (11). Aliquots of eluted material were analyzed by SDS PAGE. Fractions containing bands at 180 and 165 kDa in >95% purity (as assessed by Coomassie Blue staining) were pooled and used as immunogen. Receptor yields were 0.1–0.3 mg/100 g of liver tissue.

A New Zealand white female rabbit (Bunnyville Farms, Littletown, PA) was immunized intradermally at multiple sites with 100 μg of purified EGF receptor in incomplete adjuvant. After boosting 2 wk later with 50 μg of purified receptor in incomplete adjuvant, the rabbit was bled and the serum screened for antibody by microtiter and immunoblot techniques (42). Antibody titer remained constant for up to 10 mo.

Antibodies against the EGF receptor (anti-EGF-R) were further purified from serum by ion-exchange chromatography (58) or by affinity chromatography using purified receptor coupled to Sepharose 4B (36) and isonicotinic glycine-HCl (pH 3) elution. Ion-exchange chromatography yielded 15–20 mg IgG per 3 ml of serum while 0.2–0.3 mg of affinity-purified anti-receptor IgG was recovered per milliliter of serum applied to an EGF-receptor-Sepharose column.

Isolated Perfused Liver System

Livers from rats (150–250 g) fasted for 24 h were surgically removed and perfused in a recirculating system as previously described (17, 19). Livers were perfused at 16°C or at 37°C in the presence or absence of leupeptin (0.4 mM) for 15 min before addition of EGF (20 nM), and the rates and extents of ~2°I-EGF internalization and degradation determined from the amounts of acid-soluble and insoluble radioactivity in the perfusate and liver (20). To investigate the effects of EGF on the cellular distribution of its receptor, livers were perfused with 10 μg of EGF at 4°C, washed free of unbound ligand, and then warmed to 37°C for 4 or 15 min, or perfused with 15 μg of EGF for 15 min at 37°C. Livers were then fixed by perfusion and the receptors localized by immunofluorescence and/or immunoperoxidase cytochemistry (see below). We examined the effects of EGF on receptor degradation by perfusing livers with 0.5 mM cycloheximide (to inhibit new receptor synthesis) in the absence or presence of EGF at 37°C for 1–8 h. Biopsies were taken before and at selected times after ligand addition, liver homogenates prepared, and the EGF-binding sites and receptor protein quantitated as described below.

Subcellular Fractionation of Liver Homogenates

Livers not previously exposed to EGF (called naive livers) or perfused with 15 μg of ~2°I-EGF for 2 h at 16°C were homogenized and the 12K and microsomal pellets prepared. These pellets were then resuspended and subfractionated on linear 1.0–1.20 g/cc or 1.11–1.25 g/cc sucrose gradients as previously described (20). Accessible (~ Brj 35) and total (+ Brj 35) EGF-binding activities were measured (see below) and relative amounts of receptor protein present in subcellular fractions and in fractions obtained from sucrose gradients were determined using immunoblot techniques described below.

Quantification of EGF Receptors

EGF-Binding Activity. Accessible (~ Brj 35) and total (+ Brj 35) EGF binding activities were measured in vitro on aliquots of liver homogenates (1.5–2 mg protein), subcellular fractions (~2 mg protein), and fractions obtained from sucrose gradients as previously described (20). Briefly, samples were incubated with 80 nM ~2°I-EGF (20–100,000 cpm/ng) for 60–90 min at 4°C, and the EGF–receptor complex was precipitated in 10% polyethylene glycol and collected on Whatman GF/C filters (Whatman Inc., Clifton, NJ). This ligand concentration was sufficient for saturating high-affinity receptors (see Fig. 1 in reference 20). For Scatchard analyses, 100 μl (0.2–4 mg protein) of sucrose gradient fractions were incubated with 5–500 nM ~2°I-EGF in the presence of 0.3% Brj 35. We have shown that Brj 35 is the most effective detergent for measuring total functional or high-affinity EGF receptors. Other detergents (Triton X-100, digitonin, 2-deoxycholate, Lubrol WX, and 3-[3-cholamidopropyl]dimethyl-ammonio)-1-propane-sulfonate) dramatically reduced EGF-binding in liver homogenates and were therefore inappropriate for measuring receptors (see Fig. 3 in reference 20). Brj 35 had no effect on receptor number in homogenates of freshly isolated livers and changed receptor affinity slightly (from a Kd of 8–15 nM to a Kd of 24 nM). The total number of high-affinity receptors was determined from the highest value of EGF-binding activity measured in the presence of Brj 35. To ensure maximal EGF binding, several concentrations of Brj 35 were used. 0.2–0.3% (wt/wt) being sufficient for sucrose gradient fractions and 0.5–0.8% (wt/wt) for homogenates or subcellular fractions.

Receptor Protein on Immunoblots. Aliquots of purified receptor, liver homogenate or sucrose gradient fractions were solubilized in 1% SDS without dithiothreitol at 50°C for 10 min and the polypeptides separated on 7.5% SDS polyacrylamide slab gels. The polypeptides were then electrophoretically transferred to nitrocellulose (25) and the receptor detected as previously described (42), using affinity-purified anti-EGF-R lgG followed by ~2°I-labeled protein A. The nitrocellulose was exposed to x-ray film at -70°C with an intensifying screen. The relative amount of receptor protein present was determined by scanning the developed film and integrating the area under both peaks (170 Kd and 155 Kd) using a Hewlett-Packard Integrator (Hewlett-Packard Co., Palo Alto, CA) connected to a scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). Linearity of this quantitation reached a standard curve of 4–5 different amounts of liver homogenate that covered a 10-fold range of receptor protein. Since it was difficult to standardize the many variables in this procedure, standard curves were recalculated for each gel to ensure that the band densities were within the limits of linearity.

Immunofluorescence and Immunoperoxidase Cytochemistry

Livers were fixed by perfusion with 2% paraformaldehyde/0.075 M lysine/0.001 M sodium periodate/0.037 M sodium phosphate, pH 7.4, cut into small blocks and fixed an additional 1–6 h at room temperature. Processing and indirect immunostaining of 0.5–μm frozen sections were performed as described (42) using affinity-purified anti-EGF-R lgG and rhodamine goat anti-rabbit IgG. For co-localization studies, acid phosphatase histochemistry using sodium monochromate (CMP) as a substrate (40) was done immediately before the antibody incubations. EGF receptors were visualized at the ultrastructural level using affinity-purified anti-EGF-R lgG and a horseradish peroxidase-conjugate of sheep anti-rabbit Fab according to Brown and Farquhar (9). Acid phosphatase was localized at the ultrastructural level using CMP and cemrum as described by Robinson and Karnovsky (41).
Precipitation of EGF Receptor by Immobilized Antibody

Aliquots of liver homogenates and sucrose gradient fractions were solubilized in either buffer A (20 mM Hepes, pH 7.4, 2% Triton X-100, 10% glycerol, 0.5 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM MgCl₂, 2 mM benzanilide, 10 KIU/ml Trasylol, 1 μg/ml antipain, 1 μg/ml leupeptin, 0.5 μg/ml pepstatin, and 0.02% NaN₃) or buffer B (0.025 M NaPO₄, pH 7.4, 0.5% Triton X-100, 20 mM octylglucoside, 0.3 M NaCl, 10 KIU/ml Trasylol, 1 μg/ml antipain, 1 μg/ml leupeptin, and 0.02% NaN₃) and centrifuged for 90 min at 150,000 × g. The solubilized extract was incubated with anti-receptor IgG-Sepharose 15–24 h at 4°C after the procedures of Slieker and Lane (48) or Hubbard et al. (30) with minor modifications. The beads were then washed and the bound receptor solubilized in 4% SDS/15 mM dihydrotestosterone and electrophoresed in polyacrylamide gels.

Analytical Procedures

Protein and Enzyme Assays. Protein and enzyme markers for plasma membranes (alkaline phosphodiesterase) and lysosomes (β-N-acetyl-glucosaminidase) were assayed as described (20).

Receptor Phosphorylation. Phosphorylation of the EGF receptor either in purified form or in 0.5–2% Triton X-100-solubilized liver homogenates or sucrose gradient fractions was carried out in the presence of EGF as described by Rubin and Earp (43) and analyzed on SDS polyacrylamide gels. Phosphorylated receptor from 32P-labeled homogenates was immunoprecipitated with anti-EGF-R IgG-Sepharose following the procedures of Hubbard et al. (30).

Lectin Labeling. Wheat germ agglutinin (WGA) was applied to nitrocellulose blots of immunoprecipitated receptor according to the methods of Bartles and Hubbard (4).

Results

Preparation of Antibodies against EGF Receptor

Preparations of EGF receptor purified from rat liver by affinity chromatography routinely yielded two major protein bands of 180 and 165 kD when analyzed by SDS PAGE under reducing conditions (Fig. 1). The purified receptor retained its kinase activity as measured by autophosphorylation. However, 32P incorporation was predominantly into the 180-kD band (i.e., the intact receptor), consistent with reports that the 165-kD protein is a proteolytic fragment of the receptor and does not contain the phosphorylation site(s) (12, 57).

Antibodies prepared against the receptor recognized both SDS-denatured and Triton-solubilized forms of the molecule (Fig. 1). The reaction with the SDS-denatured receptor only when reduction of disulfides was omitted. When total liver polypeptides were solubilized and separated by SDS PAGE, then transferred onto nitrocellulose, the antibody bound to only two polypeptides, whose apparent molecular weights (Mr of 170,000 and 155,000) were identical to those of the nonreduced purified receptor (Fig. 1A). The abundant Coomassie Blue-staining protein(s) at Mr of 160,000–170,000 did not represent the EGF receptor and were not recognized by our antibodies, since they resided in vesicles that could be separated from receptor-containing vesicles on sucrose gradients (results not shown). When solubilized liver homogenates were labeled with 32P-ATP, a single band at 180 kD was immunoprecipitated. The incorporation of 32P into the 180-kD protein recognized by our antibody was stimulated in the presence of EGF. These results demonstrated that our polyclonal antibody specifically recognized rat liver EGF receptors.

Immunolocalization of EGF Receptors Before and After EGF Exposure

Recently, we reported that EGF moved through three morphologically distinct compartments (i.e., Endo I, II, and III) enroute to lysosomes (20). Biochemical evidence suggested that high-affinity EGF receptors were internalized with kinetics similar to those of the ligand and were present on sucrose gradients in vesicles of identical density to those containing internalized ligand. However, the receptor appeared to escape lysosomal degradation, since three times more EGF was internalized than the number of high-affinity receptors present (20). Utilizing anti-EGF-R antibodies and immunofluorescence (Fig. 2) as well as immunoperoxidase localizations (Fig. 3), we traced directly the pathway taken by the receptor during ligand uptake and degradation.

In the absence of EGF, receptors were localized along the microvilli of the sinusoidal front and along the lateral surface of hepatocytes (Figs. 2b and 3a). We could not detect any fluorescent labeling or peroxidase reaction product at the bile canalicular surface or inside the cell. In the presence of EGF and at times when ligand had not yet reached lysosomes (4 and 15 min), there was a progressive loss of receptors (detected as a decrease in fluorescence) at the cell periphery and the appearance of EGF receptors in more internal regions (Fig. 2). At the electron microscope level, receptors were first observed in vesicles and tubules near the sinusoidal front (Fig. 3b). The apparent molecular weights for the rat liver EGF receptor as analyzed by SDS PAGE are 170 kD + 155 kD and 180 kD + 165 kD under nonreducing and reducing conditions, respectively. Nonreducing SDS PAGE is necessary when the receptor is identified and quantified on immunoblots.
Figure 2. Immunofluorescence localization of EGF receptors in livers before and after exposure to EGF. Livers were either perfused fixed in situ (a and b) or perfused at 4°C in the presence of EGF, warmed to 37°C for 4 min (c) or 15 min (d), and then fixed. 0.5-μm sections were then incubated with affinity-purified anti-EGF-R IgG followed by rhodaminated goat anti-rabbit IgG as described in Materials and Methods. (a and b) Before EGF exposure, fluorescence (b) was localized at the sinusoidal and lateral surfaces of hepatocytes. No labeling was observed at bile canaliculi (arrowheads) or within the cell. (a) Corresponding phase contrast micrograph. (c and d) EGF internalization resulted in a progressive loss of cell surface labeling that coincided with the intracellular accumulation of fluorescence in small discrete areas near the nucleus (N). SL, sinusoidal lumen. Bar, 10 μm.

3b). These receptor-positive structures resembled coated vesicles and peripheral endosomes (20). Between 4 and 15 min after EGF internalization, the number of receptor-positive vesicles near bile canaliculi increased. These vesicles (Fig. 3, c and d) were morphologically identical to the lipoprotein-containing vesicles or multivesicular structures (i.e., multivesicular endosomes, MVE) that contained EGF conjugated to horseradish peroxidase (EGF-HRP) (Fig. 3 e). Smaller vesicles and tubules near the bile canaliculus that contained internalized ligand (EGF-HRP) were receptor-negative (compare Fig. 3, c and d to 3 e).

Effects of EGF on the Turnover of EGF Receptor in the Perfused Rat Liver

In an earlier study, we reported that the number of ligand molecules internalized and degraded by hepatocytes exceeded the total number of functional receptors in these cells. We postulated that either receptors were recycled or inactive receptors were recruited. To establish whether receptors escaped degradation and thus were available for re-utilization, we quantified the amount of receptor protein and EGF-binding activity present in cell homogenates at various times.
Figure 3. Immunoperoxidase localization of EGF receptors in livers before and after exposure to EGF (a–d) and visualization of ligand-positive vesicles by EGF-HRP cytochemistry (e). Naive livers obtained from untreated rats (a) and isolated livers perfused in the presence of EGF for 15 min (b–d) were fixed, frozen, sectioned, and incubated with affinity-purified anti-EGF-R IgG followed by sheep anti-rabbit Fab conjugated to horseradish peroxidase as described in Materials and Methods. (a) In the absence of EGF, the receptor was distributed along both the sinusoidal (SF) and lateral (LS) surfaces of hepatocytes. (b–d) By 15 min, the receptor was located in vesicles at the cell periphery and near the Golgi/lysosome region of the cell. At the periphery, receptor was visualized in structures resembling coated pits and vesicles (small arrowheads) and in larger vesicles (arrows), while in more internal regions, receptor-positive multivesicular endosomes (MVE) were observed. (e) An isolated perfused liver at 37°C was exposed to EGF-HRP for 10 min and the ligand-containing vesicles identified, by the presence of peroxidase reaction product, as previously described (20). MVEs containing EGF were morphologically identical to receptor-positive vesicles. However, the numerous smaller vesicles and tubules that contained ligand (arrowheads) were receptor-negative. Ly, lysosome; Go, Golgi apparatus. Bar, 0.5 μm.

After exposure of perfused livers to EGF.

Livers were perfused in the presence or absence of EGF under conditions that inhibited protein synthesis (i.e., 0.5 mM cycloheximide) (32), and the amount of EGF receptor present in homogenates prepared from biopsies taken at selected times was quantified on immunoblots. A representative autoradiogram is presented in Fig. 4A. Addition of EGF to the perfused rat liver resulted in a rapid loss of receptor protein and a decrease in its half-life from 8.7 to 2.5 h (Table I). The half-life of EGF-binding activity also decreased from 11.2 to 4.5 h upon ligand exposure. However, the loss of high-affinity EGF-binding sites was not coincident with EGF degradation. In fact, more ligand was degraded in 8 h than number of high-affinity binding sites lost (Table I).
Effects of Temperature and Leupeptin on the Fates of EGF and Its Receptor

The results described above demonstrated that EGF increased the rate of disappearance of receptor protein. Since we had previously shown that ligand was degraded within lysosomes (20), we next determined whether the EGF-induced loss of receptors was also affected by inhibitors of the lysosomal pathway (17, 18). EGF was added to livers perfused under conditions that inhibited fusion of endosomes with lysosomes (16°C) or lysosomal proteolysis (37°C plus leupeptin) and the dynamics of both ligand and receptor were followed (Table II and Figs. 4 B and 5).

At 16°C, internalization of EGF was limited to amounts equivalent to the number of initial high-affinity receptors measurable in vitro (Table II). In addition, EGF degradation was completely inhibited, and the reduction of hepatocyte receptor protein that was seen upon EGF exposure at 37°C was not evident at this low temperature (Table II and Fig. 4 B). When the subcellular distribution of sequestered 125I-EGF from livers perfused at 16°C was examined, 18% of the homogenate radioactivity sedimented with the lysosome-rich 12K pellet and 74% with the microsomal pellet. Sucrose gradient analysis of the latter fraction indicated that >75% of the 125I-EGF was in low density vesicles (Fig. 5). Morphological examination of livers exposed to the cytochemical tracer EGF-HRP at 16°C confirmed that the ligand was present in endosomes, both peripheral and internal tubular elements (morphology not shown). Next, high-affinity EGF binding sites were measured in gradient fractions from a liver perfused with EGF at 16°C for 2 h. These receptors were lost from an accessible pool at the plasma membrane and quantitatively recovered as a latent pool in vesicles that equilibrated on sucrose gradients at densities identical to ligand-containing vesicles (Fig. 5). We reported a similar redistribution of EGF receptors upon addition of ligand to perfused liver at 37°C for 10-15 min (Figs. 9 and 10 in reference 20). Immunoperoxidase cytochemistry on 16°C livers revealed that the receptor was present in endosomes that were structurally identical to those containing EGF-HRP (morphology not shown).

When the effects of leupeptin on ligand and receptor dynamics were examined, we found that this inhibitor had no effect on EGF uptake but inhibited the release of acid-soluble radioactivity by as much as 80% (Table II). As a result, 460,000 molecules of EGF accumulated in the leupeptin-treated hepatocyte, as compared with 200,000 molecules in the untreated cell at steady state. In addition, leupeptin completely blocked the ligand-induced loss of receptor protein (Fig. 4 B). We have previously reported that EGF accumulated

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* Livers were perfused in the absence and presence of 20 nM EGF at 37°C with medium containing 0.2 mM cycloheximide. At selected times, 0.3 g biopsies were excised, homogenized, and the number of high-affinity receptors and the relative amounts of receptor protein determined by EGF binding and immunoblot analysis, respectively, as described in Materials and Methods. Any potential problems introduced by the presence of sequestered EGF were eliminated by the assay conditions. This was accomplished using 125I-EGF of 10-fold higher specific activity and at an amount that exceeded that of the sequestered ligand by a factor of ten. In addition, 75% of the sequestered EGF was released in the presence of Brij 35, thereby exposing latent receptors. The amount of ligand degraded was quantified as described in Materials and Methods. Values presented for 1 h and 8 h were a single determination while the remaining values were averages of 2-4 measurements.

$^*$ The amount of ligand degraded in excess of the number of high-affinity receptors lost over a given time period (e.g., t = 1 h-8 h) was calculated as follows: ligand degraded at time t = (340,000 - high-affinity receptors remaining at time t).
Two Distinct Pools of EGF Receptors

The perfused liver internalized and degraded ligand well in excess of total high-affinity receptors (990,000 molecules of EGF degraded versus 340,000 EGF-binding sites/hepatocyte), even in the presence of cycloheximide (Table I). Since enhanced receptor degradation occurred under these conditions, it was unlikely that receptor re-utilization could alone be responsible for the continued uptake of EGF. Therefore, we utilized an immunological approach to explore the possibility that the hepatocyte contained more than the 300,000-340,000 high-affinity receptors measured at 80 nM EGF in our in vitro binding assay.

We had shown that high-affinity EGF-binding activity in naive livers co-migrated on sucrose gradients with conventional plasma membrane markers (Fig. 5). However, we found the distribution of receptor protein (155 kD + 170 kD) on sucrose gradients of subcellular fractions to be different (Fig. 7). 70% of the high-affinity EGF-binding activity found in both the 12K and 100K fractions equilibrated in vesicles with densities of 1.14-1.17 g/cc, which was coincident with the distribution of receptor protein (155 kD + 170 kD) recently shown to be exclusively in the sinusoidal and lateral surfaces of hepatocytes (30). In contrast, vesicles containing protein that reacted with our anti-EGF-R antibody distributed in 2-3 regions of the sucrose gradients. Less than 50% of the total immunoreactive protein recovered from either the 12K or 100K gradient was in vesicles that equilibrated with plasma membranes on sucrose gradients. Of the total immunoreactive protein present in the 12K fraction, 23% equilibrated at densities of 1.08-1.12 g/cc and 15% at 1.18-1.21 g/cc, and in the 100K fraction, 45% equilibrated at 1.08-1.12 g/cc. A 5-10-fold higher ratio of receptor protein-to-binding activity (i.e., lower specific activity) was detected in densities 1.08-1.12 g/cc as compared with that found at 1.14-1.17 g/cc (i.e., plasma membrane).

Our inability to measure EGF binding in the lower density
region of the gradients could have been due to latency of the receptors and resistance of the vesicles they reside in to detergent (i.e., Brij 35) permeabilization, or to the presence of a receptor with low affinity for EGF. All of the receptors in plasma membrane vesicles were accessible to EGF (Fig. 5), while ~70% of the EGF-binding activity measured in the lower density vesicles displayed latency, ruling out the first possibility (Fig. 8). We next characterized EGF binding to the two types of receptors by Scatchard analysis in the presence of 0.3% Brij 35 (Fig. 8). The pool of receptors at 1.14-1.17 g/cc exhibited a single high-affinity binding site with a $K_d$ of 12 nM. This value was similar to that measured in liver homogenates (20). A curvilinear Scatchard plot was observed for the receptors present at 1.08-1.12 g/cc with a majority of these receptors expressing a lower affinity ($K_d \sim 200$ nM) for EGF. These results demonstrated the existence of two pools of receptors in hepatocytes having different affinities for EGF and residing in two biochemically distinct vesicles.

**Comparison of EGF-Binding and Receptor Protein Kinase Activities with Immunoreactivity**

Human EGF receptor contains a ligand binding domain and a protein kinase domain that are separated by a transmembrane region of 23 amino acids (55). We showed that the EGF receptor purified or immunoprecipitated from liver homogenates had the expected protein kinase activity. Having identified two pools of receptors with different affinities for EGF, we next compared the protein kinase activities of the two receptor forms as measured by autophosphorylation. Low- and high-affinity receptors were solubilized in 0.5% Triton X-100 from their respective regions of the sucrose gradients and incubated with $[\gamma-^{32}P]ATP$ in the absence and presence of EGF. The results presented in Fig. 9 show that both forms of the receptor exhibited EGF-dependent autophosphorylation. Phosphorylation of receptors solubilized from either the 1.08-1.12 g/cc or 1.14-1.17 g/cc density regions was enhanced 5-10-fold by ligand. An EGF concentration of 300-400 nM was necessary for half maximal stimulation of both receptors, with maximal phosphorylation occurring at 4 $\mu$M EGF. The amount of $^{32}$P incorporated was proportional to the amount of receptor protein present as quantified on immunoblots. These results demonstrated that under our assay conditions, both low- and high-affinity receptors bound EGF and had similar levels of EGF-stimulated protein kinase activity after solubilization in Triton X-100.
Biochemical Comparison of the Two Receptor Forms

Since the low- and high-affinity forms of the EGF receptor could be segregated on sucrose gradients, we were able to compare several of their biochemical characteristics.

The fact that our antibody recognized both receptor forms suggested that there was some homology in their peptide structures. To further evaluate the extent of homology, we subjected both receptor forms to peptide map analysis. Tryptic (Fig. 10A) and chymotryptic (not shown) digests of the molecules immunoprecipitated from the two different vesicles yielded identical peptide maps. Thus, no major differences could be detected.

The apparent molecular weight and amount of the cryptic receptors suggested that they were not biosynthetic precursor forms. Nonetheless, we examined this possibility by determining whether sialic acid residues were present, as evidence for presence of complex oligosaccharides on the cryptic receptors. Equal amounts of immunoprecipitated receptor as quantified on immunoblots were resolved by SDS PAGE, transferred to nitrocellulose, and subsequently labeled with 125I-WGA using an enhancement method recently reported by our laboratory (4). WGA labeled both forms of the receptor to a similar extent and the labeling was completely inhibited by prior treatment with neuraminidase (Fig. 10B).

Discussion

In a previous paper, we showed that both ligand and high-affinity EGF-binding sites were internalized into endocytic vesicles upon exposure of hepatocytes to EGF. EGF was then degraded within lysosomes, but at a rate faster than the loss of high-affinity receptors. At that time, we proposed that either EGF receptors escaped lysosomal degradation and were re-utilized or that additional inactive receptors were recruited for further rounds of ligand internalization. In the present study, we utilized polyclonal antibodies prepared against rat liver receptor to examine these two possibilities. We have found that the EGF receptor protein is degraded more rapidly as a consequence of exposure to EGF, that it is degraded in lysosomes, and that continued uptake of ligand is not due entirely to receptor recycling. In addition, we have identified in the hepatocyte a pool of ~300,000 low-affinity receptors that may account for the continued uptake of EGF.

Fate of the Receptor after Exposure of Cells to EGF

Krupp et al. (34), Stoscheck and Carpenter (52), and Beguinot et al. (6) have shown that receptor degradation was enhanced...
when A431 cells, human foreskin fibroblasts, and KB cells
were exposed to EGF. We have observed similar results when
EGF was perfused through an isolated liver. It has been
suggested that receptors are degraded along with ligand by
lysosomal proteases, but the published evidence is not de-
finitive. Based on the finding that receptor degradation was
inhibited when cells were exposed to the weak bases, meth-
yamine and chloroquine, several investigators have proposed
that degradation occurred within lysosomes (14, 52). Al-
though these amines elevate lysosomal pH, they also affect
pre-lysosomal events by altering endosomal pH (37). It has
been reported that NH₄Cl prevents the transfer of asialo-
glycoproteins (26) and EGF (39) into lysosomes. In KB cells
previously treated with EGF, Beguinot et al. have traced the
movement of EGF receptors from the cell surface to structures
they identified as lysosomes based on morphological criteria
(6). Again, the presence of receptors in acid phosphatase-
positive vesicles was not demonstrated.

In this paper, we have presented morphological and bio-
chemical evidence suggesting that the ligand-induced loss of
hepatocyte EGF receptors was due to hydrolysis in lysosomes.
Within 15 min of EGF internalization, we found receptors in
MVEs. Wall et al. have reported that structures containing
lactosaminated ferritin and resembling MVEs could be nega-
tive or positive for aryl-sulfatase activity, suggesting that
MVEs were immediate precursors to lysosomes (56). Geuze
et al. proposed that ASGP receptors were recycled via small
tubules while ASGP-containing MVEs fused with lysosomes
(24). Thus, the detection of EGF receptors in MVEs is con-
sistent with, but not definitive of, lysosomal degradation.
Therefore, we investigated the effects of specific inhibitors of
the lysosomal pathway on the EGF-induced loss of receptors.
These inhibitors included low temperature (<20°C), which
prevents endosome/lysosome fusion (18), and leupeptin,
which inhibits lysosomal cathepsins B, H, and L (3), yet has
no effect on ligand entry into lysosomes (17, 20). Both con-
ditions inhibited EGF-induced loss of receptors, and leupeptin
resulted in demonstrable accumulation of intact EGF recep-
tors in acid-phosphatase structures. All of these results provide
strong evidence that ligand and receptor are transported along
the same pathway to lysosomes. However, from these results
we cannot conclude that every receptor that bound ligand at
the cell surface was transported to lysosomes and immediately
degraded. It is possible that some fraction of the receptors was
recycled.

Proteinase inhibitors and lysosomotropic agents have been
used to identify the site(s) of degradation of other integral
plasma membrane proteins. Leupeptin and chloroquine had
no effect on the steady-state turnover of insulin receptors in
chick liver cells (33) but efficiently inhibited the degradation
of acetylcholine receptors in cultured muscle cells (35). These
results suggest that at least two different degradative pathways
(i.e., nonlysosomal and lysosomal) exist for the turnover of
plasma membrane proteins. In addition, it is interesting that
EGF receptors are apparently not re-utilized but degraded
within lysosomes, while insulin receptors are re-utilized (22)
and degraded by a nonlysosomal mechanism. Further exper-
iments will be necessary to substantiate this interesting cor-
relation.

The dissociation of receptor and ligand within an acid
environment has been related to receptor recycling. That is,
for those ligand-receptor complexes that dissociate under
acidic conditions, the receptor apparently escapes lysosomal
degradation and is re-utilized (8). Consistent with such a
mechanism is the behavior of human choriongonadotropin-
receptor complexes, which are stable at pH ~4 and have been
shown to enter lysosomes where ligand and receptor are
degraded (1). EGF dissociates from its receptor at pH 5.0 (20),
the approximate pH measured for endosomes (37, 54). Yet
EGF receptors, apparently unoccupied, are transported along
with ligand to lysosomes. Since a majority of the receptors
are degraded, it is not presently clear why the complex is acid-
sensitive or what signals direct unoccupied receptors to lyso-
smes. However, our observations on the behavior of this
receptor raise the possibility that dissociation of ligand from
receptor may not be the only signal involved in receptor
recycling.

Two Distinct Receptor Pools

When EGF binding in liver homogenates was initially exam-
ined, ~300,000 high-affinity sites of Kd 8–15 nM were de-
tected (20). In this paper we have identified low- and high-
affinity receptors, both of which have protein kinase activity
and are structurally similar. Is it possible that low-affinity sites
could have arisen from our homogenization conditions? We
believe this to be unlikely for the following reasons. First,
discrete vesicles that could be separated on equilibrium gra-
dients contained either only high- or predominantly low-
affinity receptors, not variable mixtures. Second, we have
observed an equal distribution of receptor protein between
these two pools in six experiments, our estimate being
~300,000 in each. Third, when EGF (20 nM) is perfused
through a liver at 16°C, ligand internalization is limited to the
number of high-affinity receptors we can measure in vitro

4 The ~300,000 high-affinity receptors assayed in liver homogenates appear to
be at the cell surface, because they are accessible to EGF in vitro and co-migrate
on sucrose gradients with plasma membrane markers. Immunoblot analysis
indicates that there is an equal number or ~300,000 receptors/cell residing in
low density vesicles.
dependent protein kinase activity. It is possible that our assay
any major structural differences. Unlike the insulin and nerve
growth factor receptors (10, 13), we have not detected oligo-
addition of EGF to the perfused liver at either 37°C (20) or at
conditions (e.g., 0.3% Brij 35) may have reduced this recep-
tor's affinity. However, identical assay conditions had no
effect on the affinity of receptors that were internalized after
addition of EGF to the perfused liver at either 37°C (20) or at
16°C (Fig. 5). Therefore, the additional cryptic receptors are
either structurally different or reside in a different environ-
ment from the high-affinity receptors found at the plasma
membrane or those internalized into endosomes after EGF
exposure. Regarding the first possibility, we have not observed
any major structural differences. Unlike the insulin and nerve
growth factor receptors (10, 13), we have not detected oligo-
meric forms of the EGF receptor. Both low- and high-affinity
forms have the same apparent molecular weights of 180 kD
and 170 kD under reducing and nonreducing conditions,
respectively, and are structurally related as determined by
tryptic peptide maps. Furthermore, although the acquisition
of EGF binding occurs late in the biosynthetic pathway of the
receptor and apparently requires posttranslational glycosyla-
tion (48, 49), both low- and high-affinity receptors contain
sialic acid–terminating oligosaccharide chains. It is possible
that phosphorylation may account for the affinity differences.
12-O-Tetradecanoylphorbol-13-myristate has been reported
to stimulate the phosphorylation of receptor threonine resi-
dues (15, 31) and to inhibit high affinity EGF binding in
human cells (23, 47). Alternatively, environmental factors
may alter the receptor's conformational state and thereby its
ability to bind EGF. For example, DiPaola and Maxfield
reported that under acidic conditions the receptor undergoes
a conformational change within the membrane bilayer that
can reflect the receptor's inability to bind EGF at pH 5.6
(16). It is also possible that receptor affinity has been altered
by interaction of the protein with other membrane proteins,
lipids, and/or cytoskeletal proteins as was reported for the
nerve growth factor receptor (10, 45).

The cellular location of the pool of cryptic receptors is not
clear at this time. Low-affinity EGF receptors are found in a
membrane-bounded compartment that co-migrates on su-
crease gradients with Golgi elements and endocytic vesicles.
However, receptors in naive livers have been localized almost
exclusively to the cell surface by immunolabeling techniques
(Fig. 2). This apparent discrepancy has not been resolved, but
one possibility is that our antibody does not recognize the
cryptic pool in fixed tissue due to its orientation in the
membrane or its altered conformational state. A second pos-
sibility, that homogenization procedures may have broken or
ruptured the plasma membrane into microdomains that were
then separated on sucrose gradients, seems unlikely for two
reasons. First, CE 9, a membrane protein present exclusively
in the sinusoidal/lateral plasma membrane of hepatocytes,
did not exhibit a bimodal distribution when either a 100K
(Fig. 1) or a purified plasma membrane (see Fig. 2 in reference

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*The EGF concentration necessary for half-saturation of ligand binding at 4°C in the perfused liver was ~2 nM and in liver homogenates 8–15 nM (20).*
5) fraction was subfractionated on sucrose gradients. Second, the distribution of EGF receptor in sucrose gradients of plasma membrane subfractions coincided with that of CE 9 (i.e., a single peak at 1.13–1.15 g/cc) (data not shown).

**Dynamics of Low- and High-Affinity Receptors**

We do not have direct evidence that the low-affinity receptor functions in endocytosis nor do we know its precise relationship to the high-affinity form. The absence of high-affinity receptors in endosomes strongly suggests that EGF endocytosis is not occurring in freshly isolated livers. Therefore, it is unlikely that the cryptic receptors are a recycling pool induced by EGF internalization. However, we suggest from the following observations that the low-affinity receptors are responsive to EGF and may account for the continued uptake of ligand. The cryptic low-affinity receptor accounts for at least 50% of the total receptor protein (~600,000 receptors/hepatocyte). In the presence of EGF as much as 90% of the total receptor protein is lost, suggesting that ligand enhances the degradation of receptor in both pools. The simplest hypothesis (although certainly not the only one) is that the low-affinity receptors must be inserted into the plasma membrane where they then bind and internalize more ligand. However, the concentration of EGF in the perfusing medium (20 nM) is well below the K_d of the low-affinity receptors, necessitating activation of the receptors for them to bind ligand. This series of events appears to be inhibited at low temperatures but is not affected by leupeptin inhibition of proteolysis. That is, EGF internalization at 16°C is limited to amounts equivalent to the number of high-affinity receptors measured in vitro (300,000 receptors/hepatocyte). However, in the presence of leupeptin, 300,000 EGF molecules are taken up. Whether or not the movement of the cryptic receptors to the plasma membrane is in response to EGF is not known. It is possible, and only speculative at present, that in naive livers there is a recycling of receptors between the two pools similar to that characterized for the Fc receptor in macrophages (38). However, in the case of the EGF receptors, there is also an interconversion of the two receptor forms (high- to low-affinity and vice versa). Upon the addition of EGF, this equilibrium is altered and high-affinity receptors are diverted to lysosomes. Since >90% of all EGF receptors are lost after 8 h of continuous EGF exposure, the ultimate fate of the receptor in the presence of ligand is lysosomal degradation. Nevertheless, our current estimate of ~600,000 total receptors per hepatocyte is lower than the almost 1 × 10^6 molecules of EGF degraded in 8 h, suggesting that receptor re-utilization may be occurring.

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**References**

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