Negative Regulation of Epidermal Growth Factor Signaling by Selective Proteolytic Mechanisms in the Endosome Mediated by Cathepsin B*

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We have investigated the relevant protease activity in rat liver, which is responsible for most of the receptor-mediated epidermal growth factor (EGF) degradation in vivo. EGF was sequentially cleaved by endosomal proteases at a limited number of sites, which were identified by high performance liquid chromatography and mass spectrometry. EGF proteolysis is initiated by hydrolysis at the C-terminal Glu51-Leu52 bond. Three additional minor cleavage sites were identified at positions Arg48-Trp49, Trp49-Trp50, and Trp50-Glu51 after prolonged incubation. Using non-denaturing immunoprecipitation and cross-linking procedures, the major proteolytic activity was identified as that of the cysteine protease cathepsin-B. The effect of injected EGF on subsequent endosomal EGF receptor (EGFR) proteolysis was further evaluated by immunoblotting. Using endosomal fractions prepared from EGF-injected rats and incubated in vitro, the EGFR was lost with a time course superimposable with the loss of phosphotyrosine content. The cathepsin-B proinhibitor CA074-Me inhibited both in vivo and in vitro the endosomal degradation of the EGFR and increased the tyrosine phosphorylation states of the EGFR protein and the molecule SHC within endosomes. The data, therefore, describe a unique pathway for the endosomal processing of internalized EGF receptor complexes, which involves the sequential function of cathepsin-B through selective degradation of both the ligand and receptor.

Ligand-induced desensitization and down-regulation mechanisms are important factors in the regulation of transmembrane receptors (1), and within the receptor tyrosine kinase family, significant similarities and differences have emerged in attenuation mechanisms (2, 3). One area of research into the differing signaling outcomes of the receptor tyrosine kinases involves the intraendosomal processing of the internalized ligands and how this affects tyrosine phosphorylation of the receptor tyrosine kinases as well as their substrates (1). It has been proposed that the ligand degradation state within the endosomes contributes to both the location and specificity of downstream signaling (1, 4). Suggestive evidence has come from the differences in the levels of endosomal degradation of EGF and transforming growth factor α (TGFα) after internalization, which coincided with altered receptor trafficking (5) and was linked to the different biopotency observed for the two ligands. Recently, using the in situ liver model system for signal transduction, we have shown an alteration in insulin receptor phosphorylation and trafficking through the endosomal pathway in response to the insulin analog H2, a genetically engineered analog that displays a reduced rate of proteolysis in endosomes as compared with authentic insulin (4). Hence, the balance between net tyrosine phosphorylation and dephosphorylation in the endosome is regulated directly or indirectly by ligand proteolysis.

EGF binding to its receptor rapidly induces receptor-mediated endocytosis through clathrin-coated pits (6). Coincidentally, internalized EGF receptor (EGFR) complexes are subject to poorly defined proteolytic processes that alter their integrity and/or activity. Understanding the metabolic regulation of EGF and its receptor throughout the hepatic endosomal and lysosomal apparatus is of particular interest, as (a) in mammals, the highest concentration of the EGFR is found in liver parenchyma (7), and (b) activation of the EGFR in adult liver has been linked to organ repair through increased mitogenesis (8). Moreover, truncated EGFRs that retained their kinase activity but were internalization-defective led to enhanced mitogenesis and cellular transformation, implying a relationship between internalization and attenuation (9).

Indirect observations have suggested that EGF is proteolytically processed in endosomes to different forms that are truncated at their carboxyl termini (10). Whereas N-terminal processing can be monitored by conventional automated protein sequencing techniques using Edman degradation, no such method has been available for the study of C-terminal processing, and thus, the cleavage sites within the EGF sequence remained undefined. In this paper, we demonstrate the application of mass spectrometry for such a study.

Cathepsin B (EC 3.4.22.1) is an endosomal/lysosomal cysteine protease of the papain superfamily (11). Its main function is the degradation of peptides, proteins, and toxins that enter the endolysosomal system from outside the cell (via endocytosis or phagocytosis) (2, 3) or from other compartments within the cell (via autophagy) (11). The data presented below demon-

* This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (to F. A.) and by Grant FRM 10000320-01 from the Fondation pour la Recherche Médicale (to F. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1794 solely to indicate this fact.

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; CA074-Me, N-(t-trans-propylcarbamoyl)oxirane-2-carbonyl)-l-isoleucyl-l-proline methyl ester; E64, L-3-carboxy-2,3-trans-epoxypropionyl-leucylamide/4-quinodimethane; ENa, soluble endosomal extract; HPLC, high performance liquid chromatography; IDE, insulin-degrading enzyme; TGFα, transforming growth factor α; MS, mass spectrometry; ESI, electrospray ionization.
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Peptides, Ligand Radiodination, Antibodies, Protein Determination, and Materials—Characterization (N-terminal sequence and mass analyses) of mouse EGF (Receptor grade, Collaborative Biomedical Products) revealed that this lot (lot 907472) is an unequal mixture of mainly full-length EGF-(1–53) and two N-terminally truncated EGF fragments (EGF-(2–53) and EGF-(3–53)) in the relative proportion 64:29:7, respectively. Human insulin and porcine glucagon were purchased from Sigma. [35S]EGF (400–500 Ci/mmole) was prepared using chloramine T and purified by gel filtration on Sephadex G-50. Monoclonal horseradish peroxidase-conjugated antibody raised to phosphotyrosine was purchased from Amersham Pharmacia Biotech. Polyclonal antibody to a synthetic peptide (residues 1164–1176) of the EGFR used for immunoblotting was obtained from Dr. G. M. Di Guglielmo (McGill University, Montreal, Quebec, Canada) (12). Polyclonal antibody to a synthetic peptide (residues 1164–1176) of the EGF receptor used for immunoblotting was obtained from Dr. J. M. Bergeron (McGill University) (12). Mouse monoclonal antibody 9B12 directed against the human insulin-degrading enzyme (IDE) (13) was a kind gift from Dr. R. A. Roth (Stanford University, Stanford, CA). Rabbit anti-rat procathepsin B was used to immune deplete samples of native mature enzyme as described previously (14). Polyclonal IgG against the human insulin receptor β subunit was purchased from Upstate Biotechnology, Inc. Horseradish peroxidase-conjugated goat anti-rabbit IgG was from Bio-Rad. The protein content of isolated fractions was determined by the method of Lowry et al. (15). The ECL detection kit was from Amersham Pharmacia Biotech. Protein A-Sepharose was from Amersham Pharmacia Biotech. Human liver cathepsins B and D were from Calbiochem. CA074-Me, the methyl ester of CA074, which is a highly selective irreversible cathepsin B inhibitor, was purchased from Peptides International. All other chemicals were obtained from commercial sources and were of reagent grade.

Animals and Injections—Male Harlan Sprague-Dawley rats weighing 180–200 g were obtained from Charles River France (St. Aubin Les Elbeufs, France) and were fasted for 18 h before sacrifice. Native EGF (5 μg/100 g of body weight), native insulin (15 μg/100 g of body weight), or [35S]EGF (107 cpm) was injected into the penis vein under light anesthesia with ether. In some experiments, rats received an intraperitoneal injection of 0.5 μmol of CA074-Me in 500 μl of a 10% Me2SO/phosphate-buffered saline solution at 1 h prior to EGF injection.

Isolation of Subcellular Fractions from Rat Liver—Subcellular fractionation was performed using established procedures (4, 14, 16–19). Following injection of the appropriate ligand, animals were sacrificed, and livers were rapidly removed and minced in either isotonic ice-cold homogenization buffer containing 20 mM Tris-HCl, pH 7.4, 1 mg/ml bacitracin, 5 mM 1,10-phenanthroline, 0.1 mM E64, 2 mM N-ethylmaleimide, 4 mM NaF, and 100 μM Na3VO4. The cytosolic fraction was isolated from homogenates in 0.25 M sucrose by differential centrifugation as described previously (14, 16, 19, 20). The endosomal fraction was isolated by discontinuous sucrose gradient centrifugation and collected at the 0.25 M sucrose-1.0 M sucrose interface (4, 14, 16–19). The soluble extract from the endosomal fractions was isolated by freeze/thawing in 5 mM sodium phosphate, pH 7.4, and disrupted in the same hypotonic medium using a small Dounce homogenizer (15 strokes with the tight Type A pestle) followed by centrifugation at 300,000 × g for 30 min as described previously (4, 19, 20).

Cell-free Proteolysis and Dephosphorylation of Endosome-associated Receptors—Endosome fractions isolated in the absence of protease and phosphatase inhibitors 5 min (for insulin) or 15 min (for EGF) after the injection of native ligands were suspended at 1 mg/ml in 0.1 M KCl, 1 mM dithiothreitol, 5 mM MgCl2, and 25 mM citrate-phosphate, pH 4–7, in the presence or absence of protease and phosphatase inhibitors. Samples were incubated at 37 °C for various periods, after which degradation and dephosphorylation were stopped by adding 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2% mercaptoethanol, 10 mM EDTA, 10 μM E64 and heating at 100 °C for 2 min. Samples were then subjected to SDS-polycrylamide gel electrophoresis, followed by Western blotting to determine integrity of EGF and insulin receptors and their phosphotyrosine receptor content.

Immunoblot Studies—Electrophoresed samples were transferred to nitrocellulose blots (0.45 μm) for 60 min at 380 mA in transfer buffer containing 25 mM Tris base and 192 mM glycine. The blots were blocked by a 3 h incubation with 5% skim milk or 2% bovine serum albumin (for phosphotyrosine immunoblots) in 10 mM Tris-HCl, pH 7.5, 300 mM NaCl, and 0.05% Tween-20. The filters were incubated with primary
Cross-linking Studies—A modification of the protocol of Authier et al. (4, 14, 19) was followed. The cytosolic fraction (80 μl of protein) and soluble endosomal extract (40 μg of protein) were incubated in a final volume of 40 μl of 25 mM citrate-phosphate buffer, pH 4–8, with [125I]EGF (3 × 10^6 cpm) in the presence or absence of native EGF and protease inhibitors. After 20 min at 21 °C, cross-linking was initiated by the addition of bis(sulfosuccinimidyl)suberate to a final concentration of 0.3 mM and allowed to proceed for 15 min at 21 °C. The cross-linking reaction was terminated by the addition of 2 μl of 1 M Tris-HCl, pH 7.4. In some experiments, the subcellular fractions were immunodepleted for IDE (cytosolic fraction) or cathepsin B (soluble endosomal extract) prior to the cross-linking procedure. The hepatic fractions were incubated with either monoclonal anti-human IDE 9B12 (5 μg/ml) (for the cytosolic fraction) or with rabbit polyclonal anti-procathepsin B (1:500 dilution) (for the endosomal fraction) or with rabbit polyclonal anti-procathepsin B (1:500 dilution) (for the endosomal fraction) in 100 μl of 400 mM citrate-phosphate buffer, pH 7.4. The monomeric and polyclonal IgGs were precipitated by the addition of 50 μl of protein G-Sepharose. After rotating for 2 h at room temperature, the fractions were centrifuged for 5 min at 19,000 × g, and the resultant supernatants were subjected to cross-linking as described above. The samples were heated for 1 min at 100 °C in 1% SDS, 2% β-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis in an 8% acrylamide resolving gel according to the method of Laemmli (21).

In Vitro Proteolysis of Native Peptides—Soluble endosomal extract (about 1 ng) was incubated for various lengths of time at 37 °C with 10−6 M EGF, insulin, or glucagon in 400 μl of 50 mM citrate-phosphate, pH 4–8, in the presence or absence of protease inhibitors. The samples were then acidified with acetic acid (15%), and the resultant supernatants were subjected to reverse-phase HPLC on a Waters model 600 liquid chromatograph equipped with a model U6k sample injector fitted with a 500-μl loop and a μBondapak C18 column (Waters, 0.39 × 30 cm, 10 μm particle size). Samples were chromatographed using as eluent a mixture of 0.1% trifluoroacetic acid in water (solvent A) and 0.1% trifluoroacetic acid in acetonitrile (solvent B) with a flow rate of 1 ml/min. Elution was carried out using two sequential linear gradients followed by an isocratic elution. For EGF: an initial gradient of 2–25% solvent B (30 min), a second gradient of 25–32% solvent B (15 min), and a third isocratic elution of 32% solvent B (15 min); for insulin: an initial gradient of 2–25% solvent B (30 min), a second gradient of 25–34% solvent B (15 min), and a third
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Potential selectivity of endosomal proteases toward various internalized substrates was assessed using unmodified polypeptides to exclude potential confounding effects of radiolabeling. The ability of the ENs to degrade, at pH 5, native EGF, insulin, and glucagon was examined (Fig. 1). Incubation of the ENs with EGF and insulin resulted in a moderate generation of degradation products with 38 and 49% proteolysis, respectively, in 30 min. With glucagon, 100% degradation was detected after the same incubation period.

HPLC analysis of endosomal EGF products showed that nine peptides were produced from the native substrate (peak 9), one of which (peak 8) accounted for more than 65% of the cleavage products at any time, suggesting that it represents the major processed form of EGF within endosomes (Fig. 2).

The cleavage sites in the EGF peptides were determined by a combination of mass spectrometry and N-terminal sequence analyses (Fig. 3). According to the sequences of peptide products presented diagrammatically, two types of proteolytic activities participate in EGF degradation. The first one can be attributed to a dipeptidyl carboxypeptidase activity, which could account for the sequential generation of peptides 8 (EGF-(1–51)) and 7 (EGF-(1–49)). The second one appears to be an endopeptidase activity, which could be responsible for the generation of peptides 4 (EGF-(1–48)) and 10 (EGF-(1–50)). Other identified peptides (peaks 1–3, 5, and 6) corresponded to the remaining C-terminal residues of the above peptide products.

The effects of pH and various protease inhibitors on the EGF-degrading activity were next measured (Fig. 4). Endosomal activity peaked at pH 4–5, suggesting that an acidic protease may be involved (Fig. 4A). At pH 5, the cysteine protease inhibitors leupeptin and E64 inhibited the EGF-degrading activity by 90% (Fig. 4B). Similar results were obtained with CA074, a rapid and specific inactivator of cathepsin B (24).

Identification of an Endosomal EGF-degrading Enzyme as Cathepsin B—The inhibitory effect of CA074 on endosomal EGF-degrading activity suggested that endosomal cathepsin B may be responsible for this activity. HPLC analysis of the degradation products produced by cathepsin B (Fig. 5) revealed three proteolytic products (peaks 1, 7, and 8) with retention times identical to those seen with ENs (see Fig. 2). Mass spectrometry and N-terminal sequence analyses revealed that EGF was cleaved at the Trp49-Trp50 and Glu51-Leu52 bonds (Fig. 6), as was observed with ENs (see Fig. 3). To evaluate whether the
susceptibility of EGF to proteolysis is unique to cathepsin B, the activity of a previously identified endosomal endopeptidase, the aspartic acid protease cathepsin D (14, 25, 26) was assayed (Fig. 5). No other degradation products were observed when pure cathepsin D or a mixture of cathepsins B and D were used, suggesting that other minor cleavages observed with ENs may be produced by another endosomal endopeptidase.

We therefore used a well characterized polyclonal antibody 7183 to cathepsin B and its proform (14) to deplete cathepsin B from ENs (Fig. 7). Quantitative immunoprecipitation of cathepsin B removed 85% of endosomal EGF-degrading activity. As expected, EGF hydrolysis performed on immunoprecipitate of immunocomplex-bead eluates from anti-cathepsin B immunoprecipitations led to the generation of the major cathepsin B peptide product 8. Thus, the endosomal proteolytic activity generating EGF fragments 7 and 8 appeared qualitatively similar to that of rat hepatic cathepsin B.

Cross-linking of [125I]EGF to a 30-kDa Endosomal Binding Protein Distinct from IDE—Endosomal proteolysis of internalized polypeptides by the 110-kDa metalloprotease IDE has been suggested (27). As seen in Fig. 8A, cross-linking of [125I]EGF to a 110-kDa cytosolic protein was observed at neutral pH (lanes 4 and 5). This was competitively inhibited by 10−5 M EGF (lane 8), by bacitracin (lane 7), and by p-hydroxymercuribenzoate (lane 9), consistent with the known properties of IDE (19, 20, 28). This 110-kDa protein corresponded to IDE because it was immunodepleted (lane 10) by the well characterized monoclonal antibody 9B12 (13, 28). Comparable evaluation using the soluble endosomal extract did not reveal any protein with the molecular mass of IDE (Fig. 8B). However, at pH 6, a 30-kDa cross-linked complex was observed (lanes 1 and 4). Immunodepletion of cathepsin B by polyclonal antiserum (7183) prevented cross-linking of [125I]EGF to the 30-kDa protein (lane 2). Hence, the 30-kDa protein likely corresponds to an active form of cathepsin B.

Effect of CA074-Me on the Fate of [125I]EGF in Rat Liver in Vivo—The effect of CA074-Me treatment of rats on the metabolic fate of in vivo injected [125I]EGF was further investigated by quantitative subcellular fractionation (Fig. 9). Animals were injected intraperitoneally with either 0.15 M sodium chloride or CA074-Me 1 h prior to [125I]EGF administration and sacrificed between 2 and 90 min. [125I]EGF was retained in the liver of control animals with a maximum retention of radiolabel at 5 min and then cleared from the liver with a t1/2 of approx. 40 min (Fig. 9A, open bars). Compared with untreated rats, CA074-Me treatment revealed a 25% increase in the rate of
accumulation of radiolabel at the maximum retention time of 5 min and a significantly longer retention over the 90-min time course (Fig. 9A, closed bars). Comparable differences in the rates of accumulation of [125I]EGF into the endosomal fractions were observed between CA074-Me-treated and untreated rats.

**Metabolic Fate of Internalized EGFR within Hepatic Endosomes**—The effect of CA074-Me on endosomal EGFR signal transduction was next examined (Fig. 10). Animals were administered with an intraperitoneal injection of either 0.15 mM sodium chloride or CA074-Me 1 h prior to native EGF administration (5 μg/100 g of body weight) and sacrificed between 2 and 90 min. Following preparation of hepatic endosomes the amount of internalized EGFR, the endosomal recruitment of SH2-containing adaptor protein SHC and their phosphotyrosine content were determined using Western blot analyses. A time-dependent increase in the content of the EGFR and the two 55- and 46-kDa forms of SHC was observed in endosomal fractions at 2–15 min after EGF injection. A strong tyrosine phosphorylation of the EGFR was also observed maximally at 15 min. Following CA074-Me treatment the level of internalized EGFR and the recruitment of the 55- and 46-kDa forms of SHC within endosomes after EGF injection were more marked from 2 to 15 min and remained elevated up to 90 min. Moreover, a stronger and more prolonged phosphotyrosine content of the endosomal EGFR and 55-kDa form of SHC was observed in hepatic endosomes from CA074-Me-treated rats, as well as the presence of the tyrosine-phosphorylated 66-kDa form of SHC.

To determine whether CA074-Me alters a degradation state of the EGFR at this locus, we set out to develop a method for assaying EGFR proteolysis in intact endosomes. Therefore, we prepared endosomes at 15 min after EGF injection and the intact vesicles were incubated for various times and pH at 37 °C in an isotonic buffer mimicking the intracellular milieu (4, 16, 17) (Fig. 11). At acidic pH (4–6), a rapid loss of EGFR (Fig. 9B).

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![Fig. 10. Effect of CA074-Me treatment on the internalization of tyrosine-phosphorylated EGFR following administration of EGF in vivo.](image)
content occurred with a $t_{1/2} < 30$ s. The rapid loss of antigenicity toward $\alpha$-EGFR was entirely comparable with the results observed in the $\alpha$-PY immunoblot. A tyrosine-phosphorylated 120-kDa fragment reacting with the anti-EGFR antibody was observed at zero time.

To determine whether endosomal proteolysis of internalized EGFR was selective, the metabolic fate of the insulin receptor, another transmembrane tyrosine kinase receptor internalized into the same endocytic structures as EGF (12), and that of endosome-associated SHC were assayed using cell-free endosomes (Fig. 11). No degradation was noted for the insulin receptor $\beta$ subunit after a 30-min incubation period or for SHC, although minor degradation of the 66- and 46-kDa isoforms was detectable at pH 4 after a 2-min incubation period.

To identify the type of endosomal protease involved, freshly prepared endosomes were preincubated with various protease inhibitors before the incubation step at 37 °C (Fig. 12, lanes 2–9). Cysteine protease inhibitors (lanes 2–4 and 6–8), including the CA074-Me cathepsin B inhibitor (lane 3), reduced endosomal EGFR proteolysis by 88–97%. Inhibitors of other types of proteases were ineffective. Hence, these data implicate cathepsin B as the likely enzyme that modulates EGFR integrity within endosomes.

**DISCUSSION**

Previous studies have shown that EGF undergoes limited proteolysis at its carboxyl terminus within endosomes prior to its entry into lysosomes, where it is subsequently degraded to small peptides (10, 29). We have attempted the following: (1) to identify and to characterize EGF-degrading activity by defining the sites of ligand processing; and (2) to define the metabolic fate of the internalized EGFR throughout the endosomal apparatus.

A comparison of endosomal proteolysis of iodinated EGF, prolactin, insulin, and glucagon polypeptides using cell-free endosomes containing in vivo preloaded radiolabeled ligands demonstrated variable rates of ligand processing, i.e. high (glucagon) (16, 17), moderate (insulin) (4, 30) and low (EGF and prolactin) (31). Using an alternate approach in which hydrolysis was measured by the generation of degradation products from unlabeled peptides, we have confirmed that EGF and insulin hydrolysis, but not glucagon hydrolysis, appears to be limited.

Using a similar approach, we have previously reported that hepatic endosomes contain cathepsins B and D that process glucagon (14). Consistent with our previous study, we observed in this present work that the EGF-degrading activity within hepatic endosomes is likely to be the cysteine protease cathepsin B as indicated by the following observations: (a) the endosomal activity produced a cleavage pattern of the substrate that was very similar to that generated using pure cathepsin B; (b) the pH optimum (pH 4–5) and the inhibitor profile of the endosomal activity are similar to those of cathepsin B (32); (c) affinity-labeling experiments demonstrated the specific binding of $[^{125}\text{I}]$EGF to endosomal cathepsin B; (d) immunoprecipitation of cathepsin B from soluble endosomal extracts led to the major depletion of the endosomal activity; and (e) in situ treatment using a rapid inactivator of cathepsin B effected a major accumulation of the ligand in liver parenchyma and within hepatic endosomes.

Cathepsin B induces the cleavage of several other endocytosed polypeptide hormones, proteins, and plant toxins (2, 3, 33, 34). Thus, we have previously reported that internalized glucagon is mainly processed within hepatic endosomes by the exopeptidase activity of cathepsin B (14). Comparably, proteolysis of ricin A-chain in early endosomes at both neutral and acidic pH was primarily mediated by cathepsin B (25, 35). The participation of brain cathepsin B in endosomal-lysosomal processing to yield amyloidogenic fragments in situ from amyloid precursor proteins has also been suggested (36).

Both the endosomal activity and purified cathepsin B cleaved EGF at two distinct sites at positions Glu$^{51}$, Leu$^{52}$, and Trp$^{49}$, Trp$^{50}$. The major activity was at Glu$^{51}$, Leu$^{52}$ and accounted for 65% of the total cleavages even after a long digestion time. Thus, the pattern of peptides produced by endosomal cathepsin B is in accordance with its dipeptidyl carboxypeptidase activity observed below pH 5 (32). The resistance of EGF-(1–49) to digestion by cathepsin B and/or other endosomal proteases may result from the globular structure of the peptide product, which could prevent accessibility of the enzyme to the cleavage sites. However, an additional minor activity was present in endosomal extracts that degraded EGF to produce the EGF-(1–50) and-(1–48) fragments. This activity is not related to cathepsins B and D or to IDE, which was previously reported to be a candidate EGF-degrading enzyme (27).

Limited proteolysis of $[^{125}\text{I}]$EGF in endosomes has been identified in human fibroblasts (29) and in rat liver (10). Using hepatic endosomes, Renfrew and Hubbard (10) suggested the existence of three distinct forms of $[^{125}\text{I}]$EGF (i.e. EGF-(1–52), $[^{125}\text{I}]$EGF-(1–50), and $[^{125}\text{I}]$EGF-(1–48).
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Endosomal proteolytic activity does not utilize the insulin receptor β subunit as a substrate, it may be specific for only the internalized EGFR. The ability of cysteine-protease and cathepsin B (CA074) inhibitors to induce the in vivo and in vitro accumulation of the EGFR in the endosomal compartment implicates the cysteine protease cathepsin B in the endosomal clearance of the receptor.

Four transmembrane tyrosine kinases constitute the EGFR family: the EGFR or ErbB-1, ErbB-2, ErbB-3, and ErbB-4 (41). The EGFR family of growth factors specifically bind to the EGFR, whereas the heregulin family of growth factors associate with the ErbB-3 and ErbB-4 receptors as well as the ErbB-2/ErbB-3 receptor heterodimers. In contrast to the EGFR, all of the ErbB family members are slowly internalized in the presence of the ligand and do not exhibit ligand-enhanced metabolic turnover and down-regulation (42). Hence, alternate mechanisms exist to desensitize these surface ligand-receptor complexes, as the mitogenic potencies of EGF and heregulin are similar.

Selective ligand and receptor degradation may be an additional endosomal located mechanism further regulating signal transduction and/or sorting of internalized receptors. This was recently illustrated by sustained insulin receptor tyrosine phosphorylation and signaling observed using mutant insulin that displayed a reduced pH-dependent dissociation from its receptor within acidic endosomes and a low affinity for endosomal proteases (4). This is comparably exemplified by the intracellular fate of EGF upon occupancy by TGFα, a highly potent EGF agonist that has an identical affinity constant for binding to the EGFR but dissociates at a markedly higher pH (5). Although both EGF and TGFα have similar internalization kinetics, the targeting of the receptor for degradation in lysosomes was achieved more effectively by EGF than by TGFα (43). Whether tyrosine phosphorylation of the EGFR is enhanced in the endosome following TGFα-mediated receptor internalization is unknown. Further experiments are required to define the quantitative and qualitative differences of EGF and TGFα processing throughout the internalization pathway. This should allow us to determine whether the relevant proteases and the proteolytic events involved in the processing of both ligands within hepatic endosomes are identical or whether the existence of separate degradative pathways for EGF and TGFα may explain how these two factors exert differential effects.

Acknowledgments—We thank Pamela H. Cameron (McGill University) for reviewing the manuscript and valuable suggestions on the manuscript. We thank Dr. Richard A. Roth (Stanford University) for the kind gift of anti-IDE antibody 9B12. We thank Drs. Alain L. Servin (INSERM U510, Faculté de Pharmacie Paris XI, Châtenay-Malabry, France), John J. M. Bergeron (McGill University), and Gianni M. Di Guglielmo (Hospital for Sick Children, Toronto, Ontario, Canada) for assistance in these studies.

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Fig. 12. Effect of protease inhibitors on the endosomal EGF-degrading activity. Rat hepatic endosomal fractions were isolated 15 min after EGF administration (5 μg/100 g of body weight) and incubated in isotonic buffer at 37 °C and pH 5 in the absence or presence of 10−7 M E64, 10−7 M CA074-Me, 10 μg/ml leupeptin, 1 mM EDTA, 0.1 mM (p-hydroxymercuribenzoate), 0.1 mM (p-hydroxymercuriphenylsulfonic acid), 1 μM iodoacetic acid, or 10 μg/ml peptatin A. After a 2 min incubation, the integrity of the endosome-associated EGFR was evaluated by Western blotting. Each lane contains 20 μg of protein of the endosomal fraction. The positions of molecular mass markers are shown at the left. (−1–48), and (−1–47)) that were generated in a sequential fashion by a carboxypeptidase B-like and a trypsin-like endosomal protease. Unfortunately, in these studies, the nature of the alterations was based on comparison of the elution profiles resulting from the incubation of [125I]EGF with pure enzymes to those corresponding to endosome-associated radioactive material. Moreover, the nature of the relevant protease was deduced from the characteristics of the cleaved peptide bonds within the EGF sequence. We have confirmed here that complete degradation of EGF did not occur in hepatic endosomes. However, using a more sensitive approach, we found four C-terminally truncated forms, of which three (i.e. EGF−(1–51), −(1–50), and −(1–49)) have not been previously reported. Discrepancies between our results and those previously described may come from the fact that no direct peptide identification was performed.

A rapid EGF-induced metabolic turnover and down-regulation of the EGFR has previously been documented (37). Intracellular proteolytic activities have been suggested to be relevant under in vivo conditions because (a) incubation of intact cells in the presence of chloroquine inhibited the EGF-induced loss of the receptor (38), and (b) EGF-stimulated degradation of internalized receptor was inhibited in hepatic fractions isolated from leupeptin-treated rats (37). Furthermore, under in vitro conditions, the EGFR has been shown to be cleaved to a 94-kDa product by an acidic protease, which displays substrate specificity similar to cathepsin L and is immunoprecipitated by anti-cathepsin L antibodies (39). Finally, using pulse-chase experiments, a 125-kDa EGF fragment was identified in NIH-3T3 cells overexpressing the human EGFR, the production of which was inhibited by leupeptin and mataldehyde treatment (40). The 125-kDa species was phosphorylated on tyrosine residues in immune precipitate kinase assays and thus could be related to the 120-kDa tyrosine-phosphorylated EGF fragment observed in our endosomal fractions. With respect to parenchymal liver cells, no immunoreactive intermediate breakdown products were detected in our study other than the endosomal 120-kDa EGF product. These results suggest that in the major target organ that is enriched in EGF, the liver parenchyma, internalized EGFR is very rapidly degraded by a proteolytic process that destroys all the epitopes recognized by anti-EGFR antibodies. The data also indicate that because the
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