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Effect of Oxidative Iodination of Epidermal Growth Factor on Its Binding and Secretion by Hepatocytes

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Experiments were undertaken to determine whether the method of iodination of epidermal growth factor (EGF) affects its binding to rat liver plasma membranes and its uptake, processing, and secretion into bile by intact rat hepatocytes. EGF was iodinated using one of three oxidative reagents: chloramine T (CT), lactoperoxidase (LP), or monochloride (MC). Quantitative receptor binding studies on plasma membranes isolated from male rat livers with either CT-, LP-or MC-^{125}I-EGF indicated no significant difference in the apparent binding constants of the three preparations. To determine whether these three preparations were capable of forming a covalent-like complex with the EGF receptor, they were individually incubated with isolated plasma membranes and subjected to polyacrylamide gel electrophoresis under reducing conditions, followed by autoradiography. Each preparation formed a major radioactive protein band of ~180 kD, identified as the EGF receptor by immunoprecipitation with monoclonal anti-EGF receptor antibodies. Furthermore, even unlabeled EGF incubated with plasma membranes formed this same 180 kD band, as revealed on Western blots using anti-EGF antibody. The biliary secretion of CT-, LP-, and MC-^{125}I-EGF was compared by injecting each one into rat portal veins and measuring the total and immunoprecipitable radioactivity in bile. The amount of immunologically intact CT-^{125}I-EGF in bile was significantly greater than the others, whereas MC-^{125}I-EGF transport was significantly reduced. We conclude that the method of iodination does not affect the covalent-like binding properties of EGF. Furthermore, since unlabeled EGF displayed these same binding properties, oxidative iodination procedures per se do not account for the covalent-like association between EGF and its receptor. However, the method of iodination used did affect the intracellular transport and processing of EGF by hepatocytes. The structural modification responsible for this alteration in transport properties has yet to be determined.

Key words: EGF transport, EGF receptor, covalent EGF-receptor complex, chloramine-T, lactoperoxidase, monochloride

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Since many polypeptide hormones and growth factors retain their biological activities after radioactive labeling with [^{125}I], this method has been extensively used as a tool for studying their binding properties, uptake by target cells, and intracellular processing. It has been more or less assumed that the iodinated polypeptide interacts with its receptor and is metabolized by the target cell in the same manner as the "native," unlabeled polypeptide.

Many researchers have reported that a small portion of several iodinated ligands (insulin [1], thrombin [2], and epidermal growth factor [EGF] [2–8]) bind very strongly to their receptors, forming a "direct linkage" that exhibits some properties characteristic of covalent binding. One to five percent of the ^{125}I-EGF that binds to its plasma membrane receptor forms this "direct linkage": the receptor-ligand complex survives treatment with denaturing agents, such as SDS and 6 M guanidine-HCl, and reducing agents, such as 2-mercaptoethanol, 0.1 M dithiothreitol, or hydroxylamine [2,3,6,7], and is stable over a pH range of 3.5–11.5 [5]. However, the formation of this ^{125}I-EGF-receptor complex is also inhibited by competition with increasing amounts of unlabeled EGF and is displaceable as well [3,5], which is not characteristic of covalent associations. Therefore, since the exact nature of the binding is not known, this "direct linkage" will be referred to as "covalent-like."

Comens et al. [7] reported that this covalent-like association between ^{125}I-EGF and its receptor was an artifact caused by the harsh oxidative conditions of the chloramine T method of iodination. The amount of "direct linkage" complex formed was proportional to the reaction time and chloramine T concentration during the iodination procedure. The authors speculated that chloramine T oxidizes amino acid residues in EGF to reactive forms capable of cross-linking amino acid residues in the receptor. Other researchers have reported that EGF iodinated by the chloroglycoluril method also formed this same covalent-like complex [3,7], whereas ^{125}I-EGF prepared with lactoperoxidase (a more gentle oxidative reagent than chloramine T) did not [7].

On the other hand, Linsley et al. [4] reported (data not shown) that the covalent-like association between EGF and its receptor was not merely an artifact of chloramine T labeling, since native, unlabeled EGF also formed this same covalent-like complex with ^{35}S-methionine-labeled receptor. Therefore it is difficult to resolve whether the covalent-like properties of EGF-receptor binding are physiological or are artifactually produced by oxidative labeling methods.

This mechanism is important because the formation of this direct linkage complex may have biological consequences for the target cell. The 1–5% of EGF that participates in the formation of this linkage with its receptor may be sufficient to cause a biologically significant event. For example, the dissociation of endocytosed ligand-receptor complexes in the acidic environment of endosomes is a requisite step for subsequent entry of ligand into the normal intracellular pathway that results in lysosomal degradation [9,10]. However, that portion of EGF tightly linked to its receptor is probably unable to dissociate in acidic intracellular compartments, since it is stable at a pH as low as 3.5 [5]. It may therefore be excluded from subsequent lysosomal processing. Coincidentally, a small but significant percentage (1–5%) of ^{125}I-EGF taken up by rat hepatocytes has been shown to be transported by a direct pathway that bypasses lysosomes and results in secretion of immunologically intact EGF into bile [11].

The experiments reported here were undertaken to determine whether the

method of iodination affects: 1) the nature of the binding of ^{125}I-EGF to its receptor on the rat hepatocyte plasma membrane; and 2) the transport and metabolism of the EGF-receptor complex by the hepatocyte.

MATERIALS AND METHODS

Preparation of ^{125}I-EGF

Purified mouse EGF was purchased from Collaborative Research (Bedford, MA) and iodinated with ^{125}I-Na (Amersham, Arlington Heights, IL) by published procedures, using one of three oxidative reagents: chloramine T (CT) [12], lactoperoxidase (LP) (Enzymobeads, BioRad, Richmond, CA) [7], and monochloride (MC) [13]. The procedure for chloramine T was modified by omitting BSA from the quench reaction. The latter two compounds are milder oxidative reagents than chloramine T. The ^{125}I-labeled EGF was separated from free ^{125}I over a Sephadex G25 column, with 0.1% BSA in PBS as the elution buffer. The specific activity of the preparations ranged from 170 to 446 μCi/nmol for CT-^{125}I-EGF, 90 to 549 μCi/nmol for LP-^{125}I-EGF, and 257 to 287 μCi/nmol for MC-^{125}I-EGF. The efficiency of ^{125}I incorporation was approximately 25–55% for all three methods.

Each preparation of ^{125}I-EGF was subjected to immunoprecipitation with anti-EGF antiserum to determine if it was immunologically intact. The immunoprecipitation procedure, described in detail elsewhere [11], involved incubation of ^{125}I-EGF with rabbit anti-mouse EGF antiserum (Collaborative Research), followed by *S. aureus* with Protein A (Pansorbin, Calbiochem, La Jolla, CA). The percentage of immunoprecipitable material ranged between 75 and 90% of the total radioactivity.

Isoelectric Focusing

^{125}I-EGF prepared by each method was applied to an agarose isoelectric focusing gel (0.9% agarose), containing 12.5% sucrose, 0.6% ampholine (LKB, Bromma, Sweden), pH 3.5–5.0, and 0.4% ampholine, pH 3.5–10.0. Samples were applied near the anode. The gel was run at 15 watts for 1.5 h (maximum voltage was 1,700 volts), air-dried, and exposed at −70°C for 4 h to Kodak XR-5 film.

Plasma Membrane Binding Studies

Purified plasma membranes were prepared from excised livers of male Sprague-Dawley rats (250–300 gm) by published procedures [14]. The purity of membranes obtained with this procedure has been well established. Protein was quantitated by the method of Lowry [15], and membranes were stored at −70°C until used.

For quantitative receptor binding studies, duplicate samples of 30–50 μg of plasma membrane protein were incubated overnight with 0.2–18.0 ng of ^{125}I-EGF from each preparation at 4°C in a total volume of 170 μl 20 mM HEPES, 0.1% BSA, pH 7.4. One hundred-fold excess unlabeled EGF at each concentration was used to determine nonspecific binding, which ranged from 5 to 19%. The incubation mixture was diluted with 5 ml ice-cold PBS plus 0.1% BSA and decanted onto GF/C filters (Millipore, Bedford, MA). The filters were washed twice with 5 ml ice-cold PBS, 0.1% BSA, and their radioactivity was measured in a Beckman 8500 gamma counter. Results were plotted according to Scatchard's procedure using a graphics computer program for optimum curve fitting. The plots were not corrected for nonspecific binding. Binding constants and binding capacity were calculated using the computer pro-

gram "mod fit" [16], implemented on a Macintosh Plus computer, and were corrected for nonspecific binding.

The formation of covalent-like binding between ^{125}I-EGF and its plasma membrane receptor was qualitatively assessed. Plasma membrane protein (150–300 μg) was incubated for 1–2 h at room temperature with 15 ng of ^{125}I-EGF from each preparation in a total volume of 170 μl of PBS or 20 mM HEPES with or without protease inhibitors (1 mM PMSF, 25 mM benzamidine, 10 μg leupeptin/ml). Some incubations included 333-fold excess unlabeled EGF to use competition to demonstrate binding specificity. The membranes were washed twice and resuspended in sample buffer containing 4% SDS and 0.1 M dithiothreitol. Dithiothreitol was chosen because it is more effective at breaking disulfide bridges than 2-mercaptoethanol [17]. After boiling for 5 min, the samples were applied to 10% polyacrylamide slab gels [18]. Equal amounts of protein were applied to each lane. For determination of molecular weights, high molecular weight markers (BioRad) were applied to each gel. Autoradiograms were prepared from the gels with Kodak XR-5 film and intensifier screens.

Immunoprecipitation of EGF-Receptor Complex From Solubilized Membranes

Plasma membranes (300–600 μg) were incubated with 15 ng CT-^{125}I-EGF in 100 μl PBS for 1 h at room temperature. The membranes were solubilized by addition of 10 μl of 10% SDS, and then diluted 20-fold with PBS, containing 0.2% BSA, to a final SDS concentration of 0.05%. The EGF-receptor complexes were immunoprecipitated by adding a mixture of two IgG fractions of the monoclonal antibodies 291-3A and 291-4A, each directed against the cytoplasmic domain of the EGF receptor (gift of Dr. Alan Wells). The incubation was carried out overnight at 4°C. *S. aureus* (100 μl) with protein A was then added for an additional 2 h, and the samples were centrifuged. The immunoprecipitates were washed three times with PBS, then boiled in sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography as described above in the plasma membrane binding studies.

Identification of Unlabeled EGF on Western Blots of Liver Plasma Membranes

Plasma membranes (150 μg) were incubated with 1–2 μg of unlabeled EGF for 1 h on ice in 150 μl of 20 mM HEPES, pH 7.6, with 1 mM PMSF, 25 mM benzamidine, and 10 μg/ml leupeptin. The membranes were then boiled in sample buffer and applied to SDS polyacrylamide gels, as described above in the plasma membrane binding studies. After electrophoresis, Western blots were prepared from these gels [19]. The nitrocellulose sheets were incubated with PBS containing 5% defatted dried milk powder to reduce nonspecific binding. In a solid phase radioimmunoassay, this milk preparation showed no cross-reactivity with the rabbit anti-mouse EGF antiserum used in the next step. Even if a minute quantity of free bovine EGF were present in the milk powder, it is highly unlikely to bind to receptor on the nitrocellulose sheet, because treatment of the receptor with reducing agent severely inhibits its ability to bind EGF [20].

The nitrocellulose sheets were incubated with rabbit anti-mouse EGF antiserum (diluted 1:350 in PBS plus 5% defatted dried milk powder), followed by ^{125}I-donkey anti-rabbit IgG (10×10^6 cpm) (Amersham). As a control, to demonstrate the specificity of the immunoblot, some nitrocellulose sheets were incubated only with the sec-

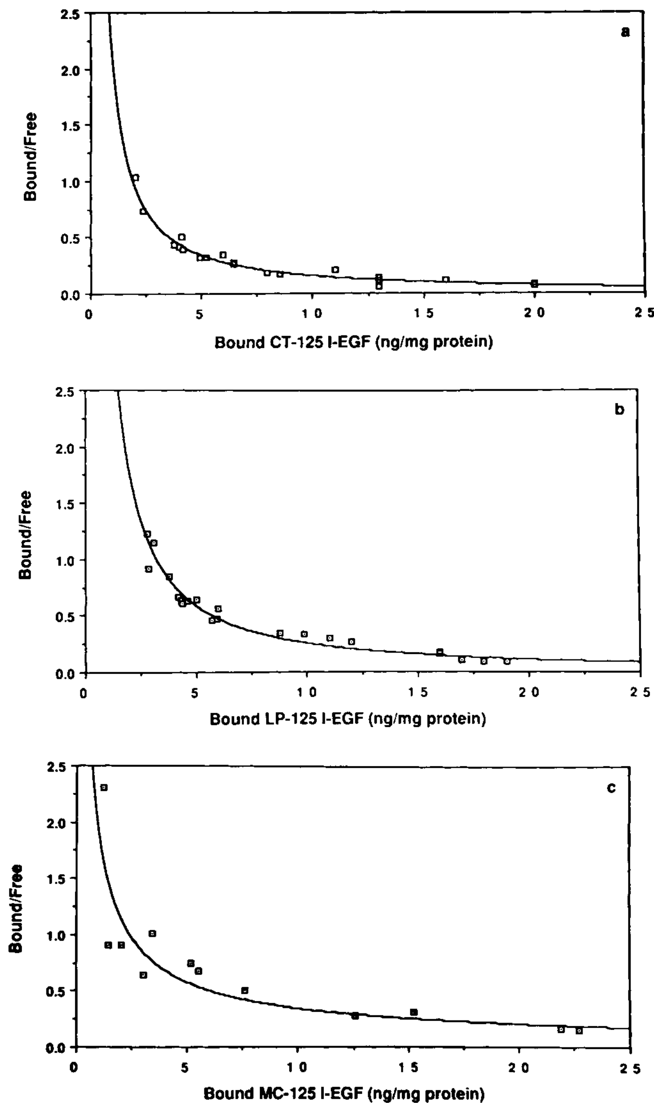

Fig. 1. Scatchard plots derived from ^{125}I-EGF binding to rat liver plasma membranes, showing the total bound radioactivity. Increasing amounts of ^{125}I-EGF (0.2–18.0 ng) were incubated with purified plasma membranes (30–50 μg). The plots are not corrected for nonspecific binding. **a:** CT-^{125}I-EGF. **b:** LP-^{125}I-EGF. **c:** MC-^{125}I-EGF.

ondary labeled antibody. Autoradiograms were prepared as described above in the plasma membrane binding studies.

Biliary Secretion of ^{125}I-EGF

Male Sprague-Dawley rats, weighing 250–300 gm, were anesthetized with nembutal, and their bile ducts were cannulated with PE10 tubing [11]. ^{125}I-EGF (~180 ng) prepared by each method, containing 8.5–17 $\times$ 10^6 cpm, was injected into the

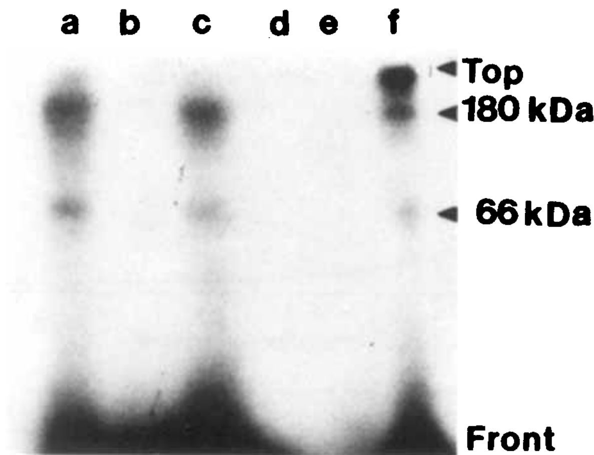

Fig. 2. Autoradiogram of a polyacrylamide gel electrophoresed under reducing conditions. Rat liver plasma membranes (150 μg) were incubated for 2 h at room temperature with 15 ng ^{125}I-EGF. The washed membranes were solubilized by boiling in sample buffer and applied to an SDS polyacrylamide gel. **Lanes a and b:** MC-^{125}I-EGF. **Lanes c and d:** LP-^{125}I-EGF. **Lanes e and f:** CT-^{125}I-EGF. **Lanes b, d, and e:** 333-fold excess unlabeled EGF was present during incubation. The radioactive band at the top of lane f represents some protein aggregates that did not properly enter the gel.

portal veins, and bile was collected on ice for 50 min. The radioactivity secreted into bile was measured, and the percentage of radioactivity immunoprecipitable with anti-EGF antiserum was determined [11].

RESULTS

Plasma Membrane Binding Studies

Quantitative binding studies were performed with CT-, LP-, and MC-^{125}I-EGF. The binding data from all three preparations generated typical binding curves on Scatchard plots (Fig. 1). The apparent binding constants ranged from 0.21 to 0.25 nM and did not appear to be significantly different. No more than one binding constant could be resolved from the existing data by the "mod fit" computer program used.

Plasma membranes incubated with CT-, LP-, or MC-^{125}I-EGF were subjected to polyacrylamide gel electrophoresis under reducing conditions. Autoradiograms (Fig. 2) demonstrated the presence of a radioactive band of approximately 180 kD with all three iodinated forms of EGF. This band corresponds in molecular weight to the previously described band representing the "direct linkage" complex of EGF and its receptor [2–8]. The band was eliminated for all three preparations when membranes were incubated with excess unlabeled EGF (lanes b, d, and e). The autoradiographic band at the dye front represents free EGF, and the band at the top of lane f represents aggregates of EGF-receptor complexes that did not properly enter the separating gel. The band at approximately 66 kD was originally thought to represent BSA, a possible contaminant of the ^{125}I-EGF preparations due to its inadvertent labeling during elution or storage. However, the presence of this same band in experiments utilizing unlabeled EGF, described below, suggested that it is probably not BSA.

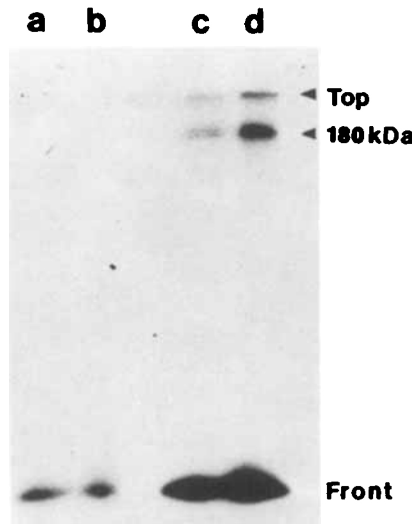

Fig. 3. Autoradiogram of a polyacrylamide gel showing immunoprecipitation of the EGF-receptor complex from solubilized rat liver plasma membranes. Plasma membranes at 300 μg (**lanes a and c**) or 600 μg (**lanes b and d**) were incubated with 15 ng of ^{125}I-EGF. The membranes were then solubilized and incubated with (**lanes c and d**) or without (**lanes a and b**) antireceptor antibody. The immunoprecipitates were boiled in sample buffer and applied to an SDS-polyacrylamide gel. The band at the top of the gel is material that did not properly enter the gel.

To identify the 180 kD band as EGF complexed to its receptor, plasma membranes incubated with CT-^{125}I-EGF were solubilized and immunoprecipitated with the monoclonal antibodies directed against the EGF receptor, and the immunoprecipitate was subjected to polyacrylamide gel electrophoresis and autoradiography (Fig. 3). The presence of the labeled 180 kD band confirmed the identity of the protein with which EGF formed the covalent-like association as the EGF receptor. Again, the band at the top of the gel was probably due to aggregates of EGF-receptor complexes not entering the separating gel.

Native, unlabeled EGF was also capable of forming a covalent-like association with its receptor (Fig. 4). Plasma membranes incubated with unlabeled EGF were subjected to polyacrylamide gel electrophoresis under reducing conditions and immunoblotted with anti-EGF antiserum. Two major protein bands demonstrated EGF binding: one at ~180 kD, and the other at ~66 kD. Since no BSA was present at any time in the preparation, the 66 kD band had to represent either a breakdown product derived from the EGF-receptor complex, or a second unrelated plasma membrane protein capable of binding EGF in the same manner.

Biliary Secretion of ^{125}I-EGF

Biliary secretion was used to monitor possible differences in the metabolism of the different ^{125}I-EGF preparations by hepatocytes. Hepatocyte transport of ^{125}I-EGF prepared by each method was assessed in vivo by injection into rat portal veins and measurement of the immunoprecipitable radioactivity secreted into bile (Fig. 5). Since lysosomal degradation products are secreted into bile, the percentage of nonim-

Fig. 4. Autoradiogram of a Western blot of unlabeled EGF bound to rat liver plasma membranes. Rat liver plasma membranes (150 μg) were incubated with 1 μg (**lane a**) or 2 μg (**lane b**) of unlabeled "native" EGF and immunoblotted with anti-EGF antiserum plus a ^{125}I-labeled secondary antibody. See Materials and Methods for details.

munoprecipitable radioactivity in bile is an indirect measure of the lysosomal degradation of ^{125}I-EGF.

Statistical comparison of the data by analysis of variance showed a significantly lower biliary secretion of radioactivity for MC-^{125}I-EGF ($P = .015$) than for CT-^{125}I-EGF over 50 min after intraportal injection. The amount of immunologically intact CT-^{125}I-EGF secreted into bile was significantly greater than the amount of LP-^{125}I-EGF ($P < 0.01$) and MC-^{125}I-EGF ($P < 0.005$) over the same time. Thus it appears that less MC-^{125}I-EGF was transported by hepatocytes, and less CT^{125}I-EGF was degraded, in comparison with each other.

Isoelectric Focusing Patterns

The isoelectric focusing patterns of CT-, LP-, and MC-^{125}I-EGF were compared (Fig. 6). The LP- and MC-^{125}I-EGF preparations each yielded a single band with the same isoelectric point. CT-^{125}I-EGF also showed this same band; however, in addition, a second, more acidic band was demonstrated.

DISCUSSION

Data from other laboratories are persuasive that chloramine T iodination procedures modify the structure of EGF:

1. Magun et al. [21] demonstrated that iodination of EGF by chloramine T generated multiple species with differing isoelectric points, only one of which had an isoelectric point of pH 4.55, corresponding to native EGF. Five major species had different binding efficiencies but were internalized by cultured fibroblasts with the same efficiency. Our finding that chloramine T iodination produced an additional form of labeled EGF is consistent with Magun's results. In addition, we demonstrated that

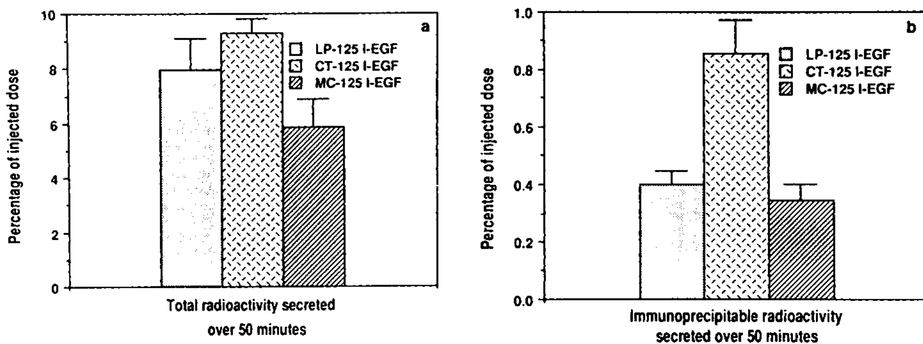

Fig. 5. Biliary secretion of ^{125}I-EGF. MC-, LP-, or CT-^{125}I-EGF (8.5–17 × 10^6 cpm) were injected into rat portal veins. Secreted bile was collected over 50 min and analyzed for both total secreted radioactivity (**a**) and radioactivity immunoprecipitable with anti-EGF antiserum (**b**). Results are presented as a percentage of the injected dose. Error bars represent standard error of the mean. n = 6 for each group.

chloramine T iodination altered the isoelectric focusing pattern of ^{125}I-EGF, relative to two other oxidative iodination procedures.

2. Comens et al. [7] demonstrated that the degree of covalent-like binding of EGF to its receptor was dependent on the length of exposure to and concentration of chloramine T during iodination.

The results reported here indicate that, regardless of the oxidative iodination method used, ^{125}I-EGF formed a covalent-like association with its plasma membrane receptor, as indicated by polyacrylamide gel electrophoresis and autoradiography. In contrast with the results of Comens et al. [7], we found that LP-^{125}I-EGF was capable of forming this same covalent-like complex. Furthermore, unlabeled EGF, not subjected to any oxidative reagent, formed this same covalent-like association as well, in agreement with the results previously reported by Linsley et al. [4]. Therefore, these data indicate that oxidative iodination per se does not cause an artifactual covalent-

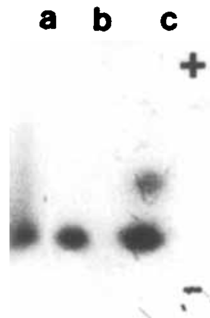

Fig. 6. Autoradiogram of freshly prepared MC-, LP-, and CT-^{125}I-EGF on an isoelectric focusing gel. Equal amounts of radioactivity from each preparation were applied to a pH 3.5–10 agarose isoelectric focusing gel. See Materials and Methods for details. **Lane a:** MC-^{125}I-EGF. **Lane b:** LP-^{125}I-EGF. **Lane c:** CT-^{125}I-EGF.

like association between EGF and its receptor. Although chloramine T iodination may modify the degree to which the covalent-like complex is formed [7], the formation of the complex is not dependent on the use of oxidative labeling methods.

Although there are presently no relevant data, it is possible that native, unlabeled EGF is already partially oxidized, and that this oxidation is responsible for the formation of the covalent-like EGF-receptor complex. The oxidation may be physiological, occurring within the organism, or it may occur during the purification of the polypeptide. If present, partial oxidation of the unlabeled starting material could account for the ability of unlabeled EGF to competitively inhibit or displace the covalent-like binding of ^{125}I-EGF.

Although the iodination procedures themselves were not responsible for the covalent-like binding between EGF and its receptor, they did affect the in vivo transport of ^{125}I-EGF by hepatocytes. Magun et al. reported differences in the binding of chloramine T-induced species of ^{125}I-EGF to fibroblasts, but no differences in their uptake [21]. We did not find any significant difference in the binding kinetics of CT-, LP-, or MC-^{125}I-EGF to rat liver plasma membranes. However, the biliary secretion of MC-^{125}I-EGF was significantly reduced, and the lysosomal degradation of CT-^{125}I-EGF was significantly diminished, relative to the other ^{125}I-EGF preparations. The iodination-induced structural modifications responsible for the alteration in transport properties have yet to be determined.

It is not known whether differences in the transport of the three preparations of ^{125}I-EGF by hepatocytes are due to differences in their type or degree of linkage with the receptor. However, it is possible that the formation of the covalent-like association does have biological consequences for the cell. For example, if that portion of the ^{125}I-EGF that forms the covalent-like EGF-receptor complex (1–5%) is, in fact, unable to dissociate once internalized into the cell, then this might conceivably result in transport via the direct (nonlysosomal) pathway and biliary secretion of intact EGF. We would then expect the intact EGF secreted into bile to remain bound to its receptor.

It should be noted that immunologically intact ^{125}I-EGF in bile has not been found in association with a 180 kD protein (unpublished observations). However, a portion of intact EGF in bile was associated with a 66 kD band (unpublished observations), analogous to the band observed in Figure 5 (with native, unlabeled EGF). A similar 66 kD protein with which EGF formed a covalent-like association was observed by Linsley and Fox [5] in 3T3 cell membranes, and was assumed to represent a breakdown product of the higher molecular weight EGF receptor.

It is tempting to speculate that the 66 kD protein in bile could represent receptor-derived protein to which EGF remains "covalently" bound. The presence in bile of immunologically intact EGF complexed to a receptor-derived protein would provide indirect evidence that the covalent-like association imparts protection from lysosomal degradation, presumably by diverting EGF into the direct transport pathway.

We have, at present, no evidence to account for differences in the intracellular processing of the three ^{125}I-EGF preparations. If the covalent-like association of EGF with its receptor does, in fact, serve to inhibit lysosomal degradation, then it is conceivable that differences in their processing could be accounted for by the degree to which they are capable of covalent-like binding with the receptor. Based on this speculation, we would predict that the EGF iodinated with the harshest oxidative reagent (chloramine T) would be the most resistant to lysosomal degradation. The data indicate that biliary secretion of intact CT-^{125}I-EGF was significantly greater than that of LP-

or MC-^{125}I-EGF. However, this explanation would not account for the reduced biliary transport of MC-^{125}I-EGF.

In conclusion, we have shown that oxidative iodination procedures alone do not account for the formation of a covalent-like association between EGF and its plasma membrane receptor. However, the method of oxidative iodination used does generate different forms of labeled EGF and does affect the intracellular transport and processing of EGF by hepatocytes.

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