Association of the Tyrosine Phosphorylated Epidermal Growth Factor Receptor with a 55-kD Tyrosine Phosphorylated Protein at the Cell Surface and in Endosomes

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Abstract. After the intraportal injection of EGF, the EGF receptor (EGFR) is rapidly internalized into hepatic endosomes where it remains largely receptor bound (Lai et al., 1989. J. Cell Biol. 109:2751-2760). In the present study, we evaluated the phosphotyrosine content of EGFRs at the cell surface and in endosomes in order to assess the consequences of internalization. Quantitative estimates of specific radioactivity of the EGFR in these two compartments revealed that tyrosine phosphorylation of the EGFR was observed at the cell surface within 30 s of ligand administration. However, the EGFR was also highly phosphorylated in endosomes reaching levels of tyrosine phosphorylation significantly higher than those of the cell surface receptor at 5 and 15 min after EGF injection. A 55-kD tyrosine phosphorylated polypeptide (pyp55) was observed in association with the EGFR at the cell surface within 30 s of EGF injection. The protein was also found in association with the EGFR in endosomes as evidenced by coprecipitation studies using a mAb to the EGFR as well as by coelution with the EGR in gel permeation chromatography. Limited proteolysis of isolated endosomes indicated that the tyrosine phosphorylated domains of the EGFR and associated pyp55 were cytosolically oriented while internalized EGF was intraluminal. The identification of pyp55 in association with EGFR in both hepatic plasma membranes and endosomes may be relevant to EGFR function and/or trafficking of the EGFR.

We and others have used subcellular fractionation and associated approaches in an attempt to delineate the components of rat liver involved in insulin, prolactin, and EGF receptor (EGFR)\(^1\) internalization in vivo (Bergeron et al., 1985; Dunn and Hubbard, 1986; Khan et al., 1986, 1989; Lai et al., 1989a,b). Past studies have revealed that after internalization into endosomes (5-15 min) the ligand, EGF, remained largely associated with the periphery of endosomes. This was revealed by EM radioautography of the distribution of silver grains from \(^{125}\)I-EGF within endosomes in situ of the placental syncytiotrophoblast (Lai et al., 1986) and for the distribution of \(^{32}\)Pi-EGF within isolated rat liver endosomes (Lai et al., 1989b). Direct visualization of internalized EGF by protein-A gold EM immunolabeling of endosomes in A431 cells has been demonstrated by Carpentier et al. (1987) and biochemical studies that evaluated the degree of ligand receptor association after polyethylene glycol precipitation of the complexes from solubilized endosomes revealed that the majority of internalized EGF within such components of liver parenchymal cells was receptor bound (Lai et al., 1989b). These studies as well as the observations that demonstrated enhanced autophosphorylation activity of the EGFR in isolated rat liver endosomes (Kay et al., 1986; Lai et al., 1989b) predicted that the phosphotyrosine content of the EGFR subsequent to internalization must remain elevated at least during transit of the receptor through the endosomal compartment(s). We have attempted to test this prediction by quantitation of the in vivo state of tyrosine phosphorylation of the receptor at the cell surface and after internalization into endosomes. Larkin et al. (1986) demonstrated the feasibility of labeling hepatic receptors such as the polymeric IgA receptor after whole animal injection of \(^{32}\)Pi. We have consequently followed this approach to label the EGFR in vivo, and in conjunction with antibodies specific to phosphotyrosine have observed that the EGFR is indeed highly tyrosine phosphorylated after internalization into endosomes after initial phosphorylation at the cell surface. Furthermore, we have observed a novel tyrosine phosphorylated protein of 55 kD (pyp55) in association with the EGFR both at the cell surface and after receptor internalization into endosomes. The orientation of the EGFR and pyp55 in isolated endosomes is such that their tyrosine phosphorylated domains are cytosolically oriented.

Materials and Methods

Materials

EGF was purchased from Collaborative Research (Waltham, MA) and insulin was a gift from the Connaught Laboratories (Toronto, Ontario). \(\text{[P}^{32}\text{P}]\)-ATP (3,000 Ci/mmol), \(\text{[P}^{32}\text{P}]\)orthophosphate (900 mCi/mmol), and Na\(^{125}\)I

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1. Abbreviations used in this paper: EGFR, EGF receptor; GE, Golgi endosomal; PM, plasma membrane.
were purchased from DuPont Canada (Mississauga, Ontario). Thin layer plates (E. Merck; 0.1 mm cellulose, 20 x 20 cm) were obtained from BDH (Montreal, Quebec). All other chemicals were from Sigma Chemical Co. (St. Louis, MO), Anachemia Canada Inc. (Lachine, Quebec), and Boehringer Mannheim (Montreal, Quebec). Sprague-Dawley rats were obtained from Charles River Ltd. (St. Constant, Quebec). For all experiments, rats were injected via the hepatic portal vein and sacrificed at various times after the injection of saturating doses (Lai et al., 1989a) of EGF (10 µg/100 g bw).

Antibodies

The hybridoma secreting anti-EGFR mAb was a gift from Dr. C.E. Chandler and was subcloned (IgG-15, BH-6) by Drs. W.A. Dunn and A.L. Hubbard (The Johns Hopkins University, Baltimore, MD). The antibodies were isolated from hybridomas as described by Lai et al. (1989b). Phosphotyrosine was conjugated to keyhole limpet hemocyanin by using 1-ethyl-3-(3-dimethyl amino)-n-pthalene-sulfonic acid and was used to raise anti-EGFR antibodies in rabbits. Antibodies were obtained by phosphotyrosine conjugated to Affigel 15 (Bio Rad, Mississauga, Ontario) column chromatography. The specificity of the antibodies was evaluated by immunoprecipitation and immunoblotting of the tyrosine phosphorylated EGFR. Thus, both immunoprecipitation and Western blotting were inhibited by phosphotyrosine but not phosphoserine or phosphothreonine (not shown).

Thin layer constituents as above was overlaid above the load zone (1:1 vol). Subsequent discontinuous gradient of 0.4 and 0.95 M sucrose containing the same buffer purified the endosome (GE) fractions. The supernatant was adjusted to 1.1 M nac in all centrifugation buffers. The PM fraction was subsequently isolated from the homogenate basically as described by Kay et al. (1986) except for the addition of 0.5 mM ATP, 10 mM ß-glycerophosphate, 5 mM Na2MoO4, 0.5 mM ATP, 20 mM Tris-HCl, pH 7.5, 100 KIU Aprotinin per ml, 0.5 M PMSF, 20 mM Na3VO4, 10 mM ß-glycerophosphate, 100 mM sodium phosphate buffer, pH 7.5. The immune complex was suspended in 1.5% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 5% ß-mercaptoethanol, and incubated for 15 min at 65°C. SDS-PAGE was carried out with a gradient gel and Coomassie blue. Resolution of the phosphoproteins on the gel were visualized by radioautography using Kodak X-OMAT X-ray film with enhancing screens. Intensity of the bands was quantified by densitometry with a Zeichn soft laser scanning densitometer interfaced with an IBM PC using a GS350 Data System (Hoffer Scientific Instruments).

In Vivo Labeling of Animals, Preparation of Plasma Membrane and Endosome Fractions, Determination of Receptor Content

Male Sprague-Dawley rats (120-130 g) received 5 mCi of [32P]orthophosphate via the portal vein. EGF also was injected via the portal vein. The livers were removed at 1 h after the injection of [32P] orthophosphate and homogenized immediately in ice-cold homogenization buffer (0.25 M sucrose, 1 mM MgCl2, 5 mM potassium iodide, 5 mM sodium orthophosphate, 5 mM Na2HPO4, 0.5 mM ATP, 2 mM benzamidine, 500 KIU Aprotinin per ml, 0.5 M PMSF, 20 mM Tris-HCl, pH 7.5) with the Dounce homogenizer (type B) to give a 15% (wt/vol) homogenate. The plasma membrane (PM) or 100,000 g for 45 min (GE) after a fourfold dilution with the homogenization buffer. To precipitate the EGFR, membranes were solubilized with 5% Triton X-100, 2.5% sodium deoxycholate, 10% glycerol, 0.15 M NaCl, 5 mM iodoacetamide, 5 mM p-nitrophosphophenylphosphate, 2 mM Na3VO4, 20 mM NaF, 10 mM ß-glycerophosphate, 50 mM sodium phosphate buffer, pH 7.5, at 4°C for 30 min and diluted 10-fold with 0.1% BSA, 0.15 M NaCl, 5 mM p-nitrophosphophenylphosphate, 100 mM sodium phosphate buffer, pH 6, then centrifuged at 50,000 g for 30 min. mAb against the EGFR (100 µg protein, IgG) was added to the supernatant (from 1 mg protein of PM or GE) and incubated for 15 min at 0°C followed by another incubation with Pansorbin for 1 h at 4°C. The immune complex was washed five times (5 min, 10,000 g) with 0.1% BSA, 0.1% Triton X-100, 0.15 M NaCl, 2 mM Na3VO4, 10 mM ß-glycerophosphate, 100 mM sodium phosphate buffer, pH 7.5. The immune complex was resuspended in 1.5% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, 5% ß-mercaptoethanol, and incubated for 15 min at 65°C. SDS-PAGE was carried out with a gradient gel and Coomassie blue. Resolution of the phosphoproteins on the gel were visualized by radioautography using Kodak X-OMAT X-ray film with enhancing screens. Intensity of the bands was quantified by densitometry with a Zeichn soft laser scanning densitometer interfaced with an IBM PC using a GS350 Data System (Hoffer Scientific Instruments).

Peptide Mapping

Peptide mapping of [125I]EGFR and [125I]pp55 was effected as follows: GE fractions isolated at 15 min after injection of EGF were solubilized with 1% Triton X-100, 0.5% deoxycholate, 10 mM Tris-HCl, pH 7.5, 10% NaCl, 2 mM Na3VO4, 10 mM ß-glycerophosphate, 10 mM sodium phosphate buffer, pH 7.5. The immune complex was recovered by centrifugation (10,000 g, 100,000 in 1 ml of 50 mM NaCl, pH 7.5. The void fraction was centrifuged at 100,000 g for 30 min after preincubation with Pansorbin for 15 min at room temperature (in order to remove nonspecific binding to Pansorbin). The supernatant was then incubated with EGFR antibody for 15 min at room temperature followed by another incubation with Pansorbin for 15 min at room temperature. The immune complex was recovered by centrifugation (100,000 g for 30 min and washed as described above. The immune complex was treated under nonreducing conditions in order to minimize contamination with IgG heavy chain with 1.5% SDS, 5% glycerol, 50 mM Tris-HCl, pH 6.8, at 65°C for 10 min and resolved by SDS-PAGE. The bands corresponding to the EGFR and pp55 were excised and tryptic peptides were obtained as follows: gel pieces were washed with 85% acetone, 5% triethylamine, 5% acetic acid, 5% water followed by 50% N-ethylmorpholine, then homogenized in 1 ml of 50 mM N-ethylmorpholine as described by Tornqvist et al. (1987). The gel suspensions were incubated with 50 µg of TPCK trypsin for 3 h at 37°C with rotation followed by incubation with another 50 µg of TPCK trypsin for 10-12 h at 37°C as described by Tornqvist et al. (1987). [125I] peptides were then resolved on cellulose plates at 500 V for 30 min in 30% formic acid for the 1st dimension, and the second dimension was chromatographed in n-butanol, acetic acid, pyridine, H2O (6:12:40:48) as described in Fig. 4. The plates were exposed to X-ray film for 1 wk (EGFR) or 30 (pp55) after staining with ninhydrin.

Limited Protease Digestion of the EGFR in Endosomes

GE fractions were isolated 15 min after the injection of 10 µg/100 g bw of EGF as described (Lai et al., 1989a,b) in the presence of the phosphate inhibitors, 2 mM NaF, 100 µM Na3VO4, and 5 mM p-nitrophosphophenylphosphate. Fractions (25 µg membrane protein) were incubated with increasing concentrations of trypsin at 0°C for 30 min in the presence or absence of Triton X-100 following which apoptosis at 2.5 times the respective concentration of trypsin was added.

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Results

Subcellular fractionation was employed to separate hepatic PM from endosome fractions of liver homogenates prepared at various times after the intraportal injection of saturating doses of EGF (10 μg/100 g bw; Lai et al., 1989a). This was followed by an assessment of EGF receptor concentration in the two fractions by quantitative immunoblotting with site-specific antibody to the EGFR as well as the level of tyrosine phosphorylation of the EGFR by in vivo labeling with \(^{32}\)P.

Evaluation of Receptor Concentration in PM and Endosome Fractions

Rapid loss of receptor from the PM and rapid concentrative internalization into endosomes was observed (Table Ia as described previously [Lai et al., 1989a]) with a 4.3-fold decrease in the concentration of EGFR in PM fractions and an 11-fold increase in receptor concentration in the endosome fraction during 15 min after the injection of EGF.

Phosphotyrosine Content of the EGFR

We next assessed the in vivo phosphorylation of the EGFR after sequential injections of \(^{32}\)P and EGF after which specific immunoprecipitation of the EGFR was done on solubilized PM and endosome fractions isolated from the same liver homogenates (Table Ib). After the injection of 5 mCi of \(^{32}\)P, the specific radioactivity of hepatic ATP was evaluated by the method of England and Walsh (1976) and found to be constant at 0.74 Ci/mol for 30–75 min after injection. Consequently, 30 min after the injection of \(^{32}\)P, EGF was injected and both PM and GE fractions were isolated from liver homogenates of rats killed at 0, 30 s, 5 and 15 min after injection. The EGFR was immunoprecipitated with mAb IgG 151-BH6 (Lai et al., 1989b) and subjected to SDS-PAGE. Maximum labeling of the receptor (170 kD) in the PM was observed at 30 s after the injection of EGF after which labeling diminished (Fig. 1A). However, in endosomes, increased labeling was found up to 15 min after EGF injection. Alkali treatment of gels (Fig. 1B) demonstrated labeling on phosphotyrosine residues for the immunoprecipitated EGFR in PM as well as in endosomes. Densitometry of the X-ray films of immunoprecipitated EGFR after SDS-PAGE and alkali treatment showed that alkali-resistant \(^{32}\)P-label in EGFRs remained nearly constant in PM fractions (when expressed per mg cell fraction protein), but increased markedly in endosomes (Table Ib). This approach enabled an estimation of the specific radioactivity of the receptor (Table Ic). Receptor-specific activity increased significantly in PM between 0 and 30 s after EGF injection but changed little thereafter. However, the specific radioactivity of the EGFR in the GE fraction did not change for the first 30 s after ligand administration despite a twofold increase in receptor concentration (see Table I, a and c). Subsequently, receptor specific activity increased eightfold between 30 s and 5 min after ligand injection and remained constant to 15 min. Of note the specific radioactivity values of the EGFR in endosomes were 2.9- and 2.3-fold higher respectively than the corresponding values for the receptor in PM at 5 and 15 min after injection.

Quantitatively similar findings were observed when experiments were evaluated by immunoblotting with antiphosphotyrosine antibodies. Immunoblotting of total cell fraction protein transferred onto nitrocellulose sheets revealed a major immunoreactive polypeptide at 170 kD whose temporal immunoreactivity in PM and endosomes was similar to that of the \(^{32}\)P-labeled immunoprecipitated EGFR (see Fig. 1). This was confirmed by immunoprecipitation studies which demonstrated that the major immunoreactive band at 170 kD was indeed the EGFR (Fig. 2B).

Identification of an EGFR-associated Polypeptide of 55 kD

In the above experiments, a polypeptide of 55 kD was also immunoprecipitated by the EGFR antibody (Figs. 1 and 2). Because \(^{32}\)P labeling of this protein persisted after alkali treatment of the gels and because it was detected by immunoblotting with phosphotyrosine antibody it is referred to as pyp55. Indeed, in both PM and endosome fractions the pyp55 band was the major antiphosphotyrosine reactive band besides that of the EGFR at 170 kD (Fig. 2). Other immunoreactive bands were observed at 47 and 64 kD (Fig. 2A). However, these proteins did not coimmunoprecipitate consistently with the EGFR (see Figs. 1 and 2B).

The immunoprecipitation studies (Figs. 1 and 2) suggested an association of pyp55 with the EGFR which was evaluated by an alternative approach (Fig. 3). Endosomal fractions,

### Table I. Ligand-mediated Changes in the Content and Specific Radioactivity of the EGFR in PM and GE Fractions

| Time (min) | PM (x 10\(^{-11}\)/mg protein) | GE (x 10\(^{-11}\)/mg protein) | PM (x 10\(^{-11}\)/mg protein) | GE (x 10\(^{-11}\)/mg protein) | PM (pmoles/100 mg cell fraction protein) | GE (pmoles/100 mg cell fraction protein) |
|-----------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|-------------------------------|-------------------------------|
| 0         | 1.59 ± 0.23                 | 0.30 ± 0.1                  | 3.2 ± 1.1                   | 1.0 ± 0.6                   | 2.0 ± 0.8                     | 3.3 ± 2.5                     |
| 0.5       | 1.02 ± 0.36                 | 0.65 ± 0.16                 | 10.0 ± 4.2                  | 3.0 ± 1.0                   | 10.2 ± 7.1                    | 4.6 ± 2.0                     |
| 5         | 0.52 ± 0.13                 | 2.13 ± 0.39                 | 6.6 ± 2.1                   | 78.4 ± 16.5                 | 12.6 ± 4.9                    | 36.8 ± 10.7                   |
| 15        | 0.37 ± 0.07                 | 3.28 ± 0.26                 | 6.2 ± 0.8                   | 123.3 ± 10.3                | 16.7 ± 4.2                    | 37.6 ± 4.4**                  |

* Time (min) after the injection of EGF.
† Receptor content (mean [n = 4] ± SD) was calculated from quantitative immunoblotting as described in Materials and Methods.
‡ \(^{32}\)P-labeled EGFR was evaluated by densitometry of radioautographs of immunoprecipitated EGFR subjected to SDS-PAGE and alkali-treatment (mean [n = 3] ± SD).
§ Specific radioactivity, calculated as the ratio of column b/column a.
‖ Significantly different from the specific radioactivity of the EGFR at 5 min in PM (P < 0.05, Student’s t test).
** Significantly different from value in PM at 15 min (P < 0.01).
isolated 15 min after the injection of EGF, were solubilized and the proteins separated by HPLC gel permeation chromatography. Eluted fractions were electrophoresed, transferred to nitrocellulose sheets, and probed with the antiphosphotyrosine antibody as well as site-specific antibodies to the EGFR. Fractions eluting at a molecular weight of ~440,000 were reactive with both sets of antibodies. Two major proteins were immunoreactive with antiphosphotyrosine antibodies; one at a molecular mass of 170 kD corresponded to the EGFR as evidenced by immunoblotting with site specific antibodies. The other protein which was immunoreactive with antiphosphotyrosine antibody had a molecular mass identical to pyp55. Whereas the EGFR was found between fractions 8-15, pyp55 was restricted to fractions 8-11 as well as in monomeric form (~60,000 in molecular weight [not shown]). This was probably due to dissociation consequent to dilution during chromatography. However, the majority of the EGFR in endosomes was of higher order structure either in association with pyp55 (fractions 8-11) or with itself (fractions 12-15, ~340,000 in molecular weight).

Two-dimensional peptide analysis was carried out on the EGFR and pyp55 after solubilization, radiolabeling with Na[^32P]I, and immunoprecipitation with anti-EGFR antibody. No overlapping peptides were found (Fig. 4) indicating that pyp55 was a distinct protein. Attempts to generate phosphopeptide maps were unsuccessful due to the low level of ^32P incorporation and the lack of sensitivity of antiphosphotyrosine immunoblots after tryptic hydrolysis of the EGFR and pyp55 (data not shown and vide infra, Fig. 5).

Pyp55 did not bind to protein A (data not shown) and therefore was not related to the heavy chain of IgG (expected molecular mass ~55 kD). Furthermore, pyp55 was not immunologically related to src as mAb MA327 (Lipsich et al., 1983) to pp60<sup>un</sup> (molecular mass 60 kD) did not immunoprecipitate pyp55 from solubilized endosomes. Neither was the protein related immunologically to the 55-kD tyrosine kinase.
phosphorylated protein identified by Baribault et al. (1989) since no reactivity was found on immunoblotting with antibodies to this protein with either immunoprecipitates of the EGFR or total endosomal proteins. Finally, phosphorylation of pyp55 was dependent on EGF. The administration of equivalent near saturating doses of insulin (15 μg/100 g bw) led to the endosomal accumulation of insulin receptors to levels similar to those of the EGFR shown in Table I. However, no association of pyp55 with the insulin receptor was found after immunoprecipitation nor was pyp55 phosphorylated after insulin administration as evaluated by Western blotting with antiphosphotyrosine antibody.

**Orientation and Localization of the In Vivo Labeled EGFR in Endosomes**

Isolated endosomes were subjected to limited proteolysis to evaluate the orientation of the EGFR and pyp55 in endosomes. Endosomes isolated 15 min after the injection of EGF were treated with increasing concentrations of trypsin at 0°C followed by immunoblotting with antiphosphotyrosine antibody. At the lowest dose of trypsin employed (0.4 μg/ml), immunoreactivity (with antiphosphotyrosine antibody) of the 170-kD EGFR as well as pyp55 was greatly diminished (Fig. 5 A; quantified in Fig. 5 B). Similar observations were found using site-specific antibody to the carboxyl-terminal tail of the EGFR (data not shown). By contrast, [32P]EGF internalized into the same endosomes was insensitive to this limited protease digestion in the absence but not the presence of detergent (Fig. 5 B). Experiments were attempted to immunolocalize directly antiphosphotyrosine antibodies on isolated endosomes by the protein–A gold technique as described by Dominguez et al. (1991). These were, however, without success presumably due to the low signal (vide infra).

**Discussion**

Our studies and those of others (Dunn and Hubbard, 1986; Kay et al., 1986; Lai et al., 1989a) have demonstrated that after EGF administration, the EGFR is rapidly internalized into hepatic endosomes. We also found that the majority of endosomal ligand (EGF) remains receptor bound even 15 min after the injection of EGF (Lai et al., 1989b). The present study was undertaken to evaluate the phosphotyrosine content of the EGFR in isolated endosomes with comparison to what was observed at the cell surface.

The radioactivity in the 32P-phosphotyrosine–labeled receptor was estimated by immunoprecipitating EGFR after the intraportal injection of 32P. Accurate determination of EGFR concentration in the subcellular fractions was achieved by quantitative immunoblotting (see also Lai et al., 1989a).
Figure 2. Immunoblot analysis of EGFR and substrates by anti-phosphotyrosine antibody. Membrane fractions (50 μg) of PM (lanes 1-4) and GE (lanes 5-8) were isolated from rats sacrificed at 0, 0.5, 5, and 15 min after the injection of EGF. (A) The fractions were subjected to SDS-PAGE and immunoblotted with antiphosphotyrosine antibody as described in Materials and Methods. (B) Endosomes (GE, 50 μg protein) were solubilized and after incubation with EGFR antibody, the immunoprecipitate was subjected to SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibody. At 15 min after the injection of EGF additional bands at 47 and 64 kD are observed in addition to the EGFR and pyp55. (C) GE and PM fractions (100 μg protein each) isolated at 0.5 and 15 min after the injection of EGF were incubated with 0.1 M sodium carbonate on ice for 30 min followed by centrifugation at 200,000 g for 30 min. The supernatants (S) and pellets (P) were subjected to SDS-PAGE followed by immunoblotting with antiphosphotyrosine antibody. The positions corresponding to the molecular masses of the EGFR and pyp55 are indicated.

using a site-specific antibody to the EGFR. 32P-phosphotyrosine content per unit receptor (i.e., specific radioactivity) was then calculated from the densitometry of 32P-labeled immunoprecipitated EGFR divided by the receptor content. The data clearly establish that ligand-dependent tyrosine phosphorylation was initiated at the cell surface with a five-fold increase in receptor specific activity observed in plasma membranes within 30 sec after the administration of EGF. Endosomal receptor specific activity was significantly greater than that of PM receptors at either 5 (P < 0.05) or 15 min (P < 0.01) after injection. However, when calculated as the fold increase in specific activity over that at zero time, receptor-specific activity in endosomes at 15 min was only slightly greater (11.4-fold increase) than that calculated for PM over the same time interval (8.4-fold increase). (This discrepancy was due to the high variation in the estimation of the low receptor concentration and low 32P-labeling of the EGFR in the GE fraction at zero time (Table I c)). Though consistent with the view that receptor phosphorylation was enhanced in endosomes our data do not exclude the possibility that receptor phosphorylation occurred only in the PM with highly phosphorylated receptors being preferentially internalized. On the other hand, within the first 30 s after EGF injection PM receptor specific activity increased five-fold whereas endosomal receptor specific activity remained similar to the low zero time level despite a twofold increase in receptor concentration in endosomes. Here there would appear to have been selective internalization of only poorly phosphorylated cell surface EGFRs. Thus the hypothesis of selective internalization appears to be a rather more complicated explanation for our data.

The yield of the PM fraction was ~14% based on the
Figure 3. Coelution of pyp55 with EGFR in gel permeation chromatography. GE fractions (250 μg protein) isolated 15 min after the injection of EGF (10 μg/100 g bw) were solubilized as described in Materials and Methods. Eluted fractions were subjected to immunoblotting with the anti-phosphotyrosine antibody (upper panel) or a mixture of site-specific antibodies against synthetic peptides corresponding to residues 1,164-1,176 (αP1 Ab) and 1,059-1,072 (αP3 Ab) of the EGFR. Vo, void volume; 440 K, elution position of ferritin; 160 K, elution position of γ-globulin. On the left is indicated the positions of the EGFR and pyp55.

receptor content of these fractions compared to that of a total particulate fraction (Lai et al., 1989a). The yield of endosomes was ~32% (calculated from the receptor content in endosomes at 15 min after injection of saturating levels of EGF (Table I of the present study and Lai et al., 1989a) as compared to that of total particulate fractions of liver homogenates (Lai et al., 1989a). EM of the PM fraction indicated a representative cell fraction consisting of all domains (sinusoidal, lateral, bile canalicular) of the hepatic cell surface (Hubbard et al., 1983; Lai et al., 1989a). This was not the case for the endosomal fraction. The endosomal components of the GE fraction consisted mainly of tubulovesicular profiles with the vesicular components of ~250–300 nm in diameter containing intraluminal lipoprotein-like particles (Lai et al., 1989b; Doherty et al., 1990). The much larger (and denser) multivesicular bodies were not found in this fraction. Indeed, the studies employing limited proteolysis of the GE fraction (Fig. 5) demonstrated that the tyrosine phosphorylated domain of the EGFR was cytosolically oriented while internalized [125I]EGF was intraluminal. Taken together with past studies showing that at this dose of injected ligand and at 15 min after injection, [125I]EGF was largely receptor bound and localized to the bounding membrane of endosomes (Lai et al., 1989b), we conclude that little if any of internalized-EGF or tyrosine-phosphorylated EGFR was sequestered within intraluminal vesicles of multivesicular bodies in the GE fraction. Other investigators have clearly demonstrated internalized EGFR within such structures (McKanna et al., 1979; Hopkins, 1990; McCune et al., 1990). It is, however, noteworthy that Carpenter et
al. (1987) have immunolocalized phosphotyrosine to the cytosolic surface of endosomes in A431 cells after the administration of EGF. Even so, A431 cells have been reported by Wiley et al. (1988) to be defective in internalization of the EGFR. Hence, the studies of Carpentier et al. (1987) may have underestimated the significance of phosphotyrosine labeling in endosomes. A431 cells have been reported to have ca. \(2 \times 10^6\) receptors per cell (Haigler et al., 1979; Krupp et al., 1982; Gamou et al., 1984). The much larger hepatocyte has less than \(10^5\) receptors per cell (Lai et al., 1989a). It was perhaps not surprising therefore that our own attempts to visualize phosphotyrosine labeling in endosomes by EM immunolabeling were unsuccessful (not shown).

In vivo labeling of the EGFR was determined after immu-

Figure 4. Two dimensional peptide maps of EGFR (A) and pyp55 (B). GE fractions were isolated at 15 min after the injection of EGF. The fractions were solubilized, dephosphorylated, and iodinated with Na\(^{125I}\) as described in Materials and Methods. After immunoprecipitation with mAb to the EGFR, the radioiodinated EGFR and pyp55 were resolved by nonreducing SDS-PAGE, extracted from the gel, and digested with TPCK trypsin. Resulting peptides were applied onto a cellulose plate equilibrated with 30% formic acid and the \(^{125I}\) labeled tryptic peptides were resolved electrophoretically in 30% formic acid, then by chromatography in n-butanol/acetic acid/pyridine/H\(_2\)O (60:12:40:48). The plates were stained with ninhydrin and were exposed to X-ray film. In each case, the origin is indicated (O). None of the major \(^{125I}\) peptides of the EGFR (A) corresponded to those of pyp55 (B). The numbers indicate the locations of the ninhydrin positive spots which were due to degraded fragments of trypsin and used to align the two maps.
Figure S. Orientation and localization in intact endosomes of the in vivo phosphorylated EGFR and pyp55. GE fractions were isolated 15 min after the injection of unlabeled EGF (10 μg/100 g bw). The fractions (15 μg membrane protein) were incubated with increasing concentrations of trypsin as described in Materials and Methods, then electrophoresed by SDS-PAGE, the proteins transferred to nitrocellulose sheets and probed with antiphosphotyrosine antibodies (A). The proportion of phosphotyrosine reactive EGFR (170 kD) (○○○○) and pyp55 (55 kD) (▲▲▲) were then estimated by densitometry (B). The proportion of protease sensitive internalized [125I]EGF in endosomes was determined on concurrent experiments carried out in the presence (■■■■) or absence (●●●●) of Triton X-100.
noprecipitation with a mAb to the EGFR. Coprecipitating with the EGFR was an associated phosphotyrosine-labeled protein (pyp55) whose tyrosine phosphorylation was EGF dependent. Pyp55 was also readily seen on immunoblotting PM and endosome fractions with antiphosphotyrosine antibodies. It was concluded that the protein was specifically associated with the EGFR in endosomes on the basis of its coprecipitation as well as by its demonstrated association during HPLC gel permeation chromatography of solubilized endosomes. The molecular weight of the EGFR:pyp55 complex was estimated by gel permeation chromatography to be ~440,000 which would be consistent with 2 mol of the EGFR and 2 mol of pyp55. The phosphoprotein pyp55 was found in association with the EGFR at initial times of activation at the cell surface, i.e., at 30 s as well as at peak times of internalization in endosomes (5-15 min). It was not possible to identify by Coomassie blue staining the amount of this protein in immunoprecipitates since it was below the limit of detection.

From their studies on the regulation of recycling of the Fc receptor, Mellman et al. (1984) have proposed that conditions favoring Fc receptor oligomerization would lead to downregulation while conditions favoring receptor monomer formation would lead to receptor recycling. The studies of Honegger et al. (1987) and Felder et al. (1990) have suggested that the tyrosine kinase activity of the EGFR in endosomes may be necessary for downregulation. Since our experimental conditions (EGF dose of 10 μg/100 μg bw) favored downregulation (Lai et al., 1989a) we suggest that a possible function of pyp55 is to regulate the oligomerization of the EGFR in endosomes in a tyrosine phosphorylation dependent manner thereby regulating receptor downregulation. Current experiments aimed at purifying pyp55 and determining its primary structure by cDNA cloning could help elucidate the significance of this EGFR-associated phosphoprotein.

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