Proc. Natl. Acad. Sci. USA
Vol. 89, pp. 8477–8481, September 1992
Biochemistry

Epidermal growth factor stimulates tyrosine phosphorylation in the neonatal mouse: Association of a M_r 55,000 substrate with the receptor

ROBERT W. DONALDSON* AND STANLEY COHEN†

Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN 37232

Contributed by Stanley Cohen, June 8, 1992

ABSTRACT **Administration of epidermal growth factor (EGF) to neonatal mice resulted in rapid tyrosine phosphorylation of a number of specific substrates in liver, kidney, lung, bladder, skin, and brain as detected by Western blot analysis of tissue homogenates with anti-phosphotyrosine antibodies. In the liver, three prominent EGF-dependent substrates of $M_r \approx$ 170,000, 120,000, and 55,000 were detected. A number of less prominent EGF-dependent substrates also were noted. Maximal tyrosine phosphorylation of pp170, pp120, and pp55 occurred within 5 min of subcutaneous injection and the levels of these phosphoproteins remained elevated for at least 45 min. Direct hepatic injection of EGF resulted in the tyrosine phosphorylation of these substrates within 60 sec of treatment. Tyrosine-phosphorylated pp170 was identified as the EGF receptor (EGFR). The tyrosine-phosphorylated pp55 substrate appeared to be associated with EGFR; both pp55 and EGFR were adsorbed to EGF-Affi-Gel, wheat germ lectin-Sepharose, and anti-EGFR antibodies bound to protein A-Sepharose. pp55 was not immunoreactive with anti-EGFR antiserum by Western blot analysis, indicating that it was not a fragment of the receptor. These results were confirmed by repeating the liver experiments using ^{32}P-labeled neonatal mice. Increased amounts of ^{32}P-labeled pp170 and pp55 were detected in anti-EGFR immunoprecipitates from liver extracts of EGF-treated animals as compared with controls. Phospho amino acid analysis of the ^{32}P-labeled phosphoproteins revealed that EGF stimulated both serine and tyrosine phosphorylation in pp55 as well as in EGFR. The neonatal mouse may be a useful model for the study of signal transduction mediated by a variety of growth factors.**

The administration of epidermal growth factor (EGF) to newborn or adult animals evokes a wide variety of morphological and physiological responses. These include such diverse effects as increased cell proliferation in skin and other organs as well as inhibition of gastric acid secretion (reviewed in refs. 1–3).

At the cellular level, the receptor for EGF (EGFR) is a 170-kDa transmembrane glycoprotein with intrinsic protein-tyrosine kinase activity. The binding of EGF to its receptor results in the activation of its tyrosine kinase activity and the phosphorylation of a number of intracellular substrates as well as the receptor itself (reviewed in refs. 4–6).

Many studies have addressed the question of the nature and function of protein substrates that are phosphorylated on tyrosine in the presence of an activating ligand. Among the substrates that have been implicated are phospholipase $C_{\gamma 1}$, GTPase-activating protein (GAP), protein-serine kinases, phosphatidylinositol 3-kinase, structural proteins such as vinculin and talin, and proteins of unknown physiological function such as lipocortin (reviewed in refs. 7–9). Almost all of these studies have employed a variety of cell culture systems to detect these putative signal-transducing molecules.

We have begun studies to examine both the normal physiological role of EGF during development and the signal transduction processes that are activated *in vivo* following the administration of EGF to neonatal mice. In this initial report, we demonstrate rapid EGF-dependent tyrosine phosphorylation of a number of proteins, including EGFR, in all organs examined. We further demonstrate EGF-dependent phosphorylation of a protein of $M_r \approx 55,000$ that is associated with EGFR. Our data on EGF suggest that the neonatal mouse may be a useful experimental system for the detection of signaling pathways employed by other growth factors.

MATERIALS AND METHODS

Materials. ND4 Swiss Webster mice were obtained from Harlan–Sprague–Dawley. EGF, EGF-Affi-Gel, polyclonal rabbit anti-mouse liver EGFR, and rabbit polyclonal anti-human A-431 EGFR were prepared as described (10). Porcine insulin was from Squibb-Novo, Princeton, NJ. Polyclonal anti-phosphotyrosine was from Zymed Laboratories. Okadaic acid was from Moana Bio Products, Honolulu. ^{125}I-labeled protein A and [^{32}P]orthophosphate were from ICN. Immobilon-P membranes were from Millipore. Nitrocellulose membranes were from Schleicher & Schuell. Prestained SDS/PAGE molecular weight standards were from BRL. Wheat germ lectin-Sepharose, protein A-Sepharose, phospho amino acids, and other reagents were from Sigma.

Preparation of Tissue Extracts. Solutions of EGF [1 mg/ml in phosphate-buffered saline (PBS)] or insulin (100 units/ml) were injected subcutaneously into neonatal mice (0.5–9.5 days old) at a dose of 10 μl/g of body weight. Mice were sacrificed by cervical dislocation or decapitation at the indicated times. Organs of interest were excised immediately and homogenized (7.5% wet weight/vol) on ice in solubilization buffer A [20 mM Hepes, pH 7.4/1% (wt/vol) Triton X-100/2 mM EGTA/2 mM EDTA/500 μM Na_3VO_4/50 μM $Na_2Mo_4O_4$, containing aprotinin (10 μg/ml) and leupeptin (10 μg/ml)]. Homogenates were centrifuged at 14,000 × *g* for 1 min and aliquots of the supernatants were heated in Laemmli sample buffer (11) at 95°C for 5 min prior to SDS/PAGE. Adsorption of the EGFR and other proteins was carried out as follows. Aliquots (500 μl) of supernatants were incubated with either EGF-Affi-Gel (100 μl) or wheat germ lectin-Sepharose (100 μl) or with antisera to either mouse liver EGFR (20 μl), or human A-431 EGFR (20 μl), or phosphotyrosine (5 μl) and mixed for 4 hr at 4°C. When antisera were

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: EGF, epidermal growth factor; EGFR, EGF receptor; PBS, phosphate-buffered saline.

*Present address: Analytical Biochemistry, Analytical Research & Development, Bristol-Myers Squibb Pharmaceutical Research Institute, P.O. Box 4755, Syracuse, NY 13221-4755.
†To whom reprint requests should be addressed.

employed, protein A-Sepharose (100 μl) was added 30 min prior to processing. The protein-bound beads were washed four times with 1 ml of solubilization buffer A and adsorbed proteins were eluted by heating the samples in Laemmli sample buffer at 95°C for 5 min.

Detection of Phosphorylated Proteins by Western Blot Analysis. Proteins were resolved by SDS/6.5% PAGE and electrophoretically transferred to nitrocellulose for 30 min at 50 V with a Sartoblot-IIS semidry transfer apparatus (Sartorius) and Sartoblot transfer buffer (48 mM Tris/39 mM glycine/1.3 mM SDS/20% methanol, pH 8.3). Nitrocellulose was blocked with 3% bovine serum albumin (fraction V) for 1 hr, probed with anti-phosphotyrosine (1:1000) or anti-mouse liver EGFR (1:250) for 1 hr, incubated with ^{125}I-protein A (10^6 cpm/ml) in SDS/Triton blot buffer (50 mM Tris, pH 7.4/150 mM NaCl/5 mM EDTA/0.25% gelatin/0.5% Triton X-100/0.1% SDS) for 1 hr, and then washed with SDS/Triton blot buffer for 30 min. Immunoreactive proteins were detected by autoradiography using Cronex film (E. I. du Pont de Nemours, Greengard, NC) or a Molecular Dynamics PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Detection and Analysis of [^{32}P]Phosphoproteins Labeled *in Vivo*. Neonatal Swiss Webster mice (4.5 days old) were injected subcutaneously with 4 mCi (148 MBq) of [^{32}P]orthophosphate 2 hr before administration of EGF. EGF or PBS was injected subcutaneously 10 min before sacrifice. Livers were excised and immediately homogenized in solubilization buffer B (solubilization buffer A containing 50 mM β-glycerol phosphate, 10 mM NaF, and 10 nM okadaic acid). Homogenates were centrifuged at 14,000 × *g* for 1 min and aliquots of the supernatant were incubated with antiserum against mouse liver EGFR and protein A-Sepharose as described above. The protein-bound Sepharose was washed eight times with solubilization buffer B, and adsorbed proteins were eluted and recovered as described above. Proteins were resolved by SDS/6.5% PAGE and electrophoretically transferred to Immobilon-P, and ^{32}P-labeled phosphoproteins were detected by autoradiography using Kodak X-AR film (Eastman Kodak). Proteins transferred to Immobilon-P were detected by staining with amido black 10B (12). Regions of Immobilon-P containing pp170 or pp55 were excised and subjected to phospho amino acid analysis as follows. Immobilon-P slices were wetted in methanol and transferred to 500 μl of 6 M HCl and heated for 60 min at 110°C. Immobilon-P was removed and the sample was concentrated by centrifugation under vacuum. Reconstituted samples containing phospho amino acid standards were spotted onto cellulose acetate plates (Eastman Kodak) and resolved by high-voltage electrophoresis at 500 V for 60 min at pH 3.5 (pyridine/glacial acetic acid/water, 10:100:1890, vol/vol). The phospho amino acid standards were identified with ninhydrin and the ^{32}P-labeled phospho amino acids were detected by autoradiography using a Molecular Dynamics PhosphorImager. The image was photographed using a Polaroid DS-34 direct screen camera and 667 film. The phosphorylated amino acids were identified by alignment with ^{32}P-labeled markers in a separate exposure.

RESULTS

Effect of Administration of EGF to Neonatal Mice on Tyrosine Phosphorylation. EGF in PBS was injected subcutaneously into neonatal mice at a dose of 10 μg/g of body weight. Control animals received injections of PBS. After 5 min, organs were excised and processed for Western blot analysis using anti-phosphotyrosine antibodies. The results are illustrated in Fig. 1 *A* (0.5-day-old mice) and *B* (9.5-day-old mice). In all organs examined (liver, kidney, lung, brain, bladder, and skin), administration of EGF resulted in an enhancement of tyrosine phosphorylation of many proteins. These included proteins of apparent molecular mass 170, 120, 90, 55, and 35 kDa (pp170, pp120, pp90, pp55, and pp35). The relative abundance of phosphorylated tyrosine in these proteins varied, depending on the organ examined. For example, the phosphotyrosine signal in pp170 was most prominent in the liver but was detected, at varying levels, in all organs; pp55 was readily detectable in liver, kidney, lung, and skin but not in brain; pp35 was most prominent in the kidney; pp120 was most prominent in the lung and appeared to be a mixture of proteins. Enhanced tyrosine phosphorylation also was detected in the testes of EGF-treated animals (data not shown). In control experiments the phosphotyrosine signal detected by Western blot analysis was completely abolished by preincubation of the anti-phosphotyrosine antibodies with soluble phosphotyrosine (data not shown).

Time Course of Tyrosine Phosphorylation in Liver. Neonatal mice (6.5 days old) were sacrificed and processed at the indicated times following the subcutaneous injection of EGF. At the EGF dose used, maximal enhancement of tyrosine

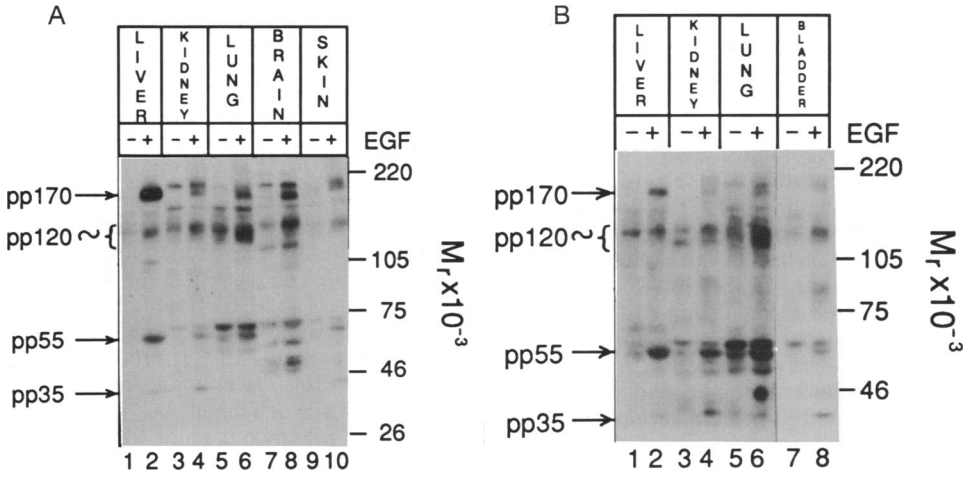

FIG. 1. EGF-dependent tyrosine phosphorylation in various organs of the neonatal mouse at 0.5 day (*A*) or 9.5 days (*B*) of age. EGF (1 mg/ml in PBS) or PBS was injected subcutaneously at a dose of 10 μl/g of body weight. Mice were sacrificed after 5 min of treatment. Organs of interest were excised and processed. Aliquots of the clarified homogenate (30 μl) were resolved by SDS/PAGE and transferred to nitrocellulose. Phosphotyrosine was detected by Western blot analysis using antibodies to phosphotyrosine (1:1000). The organs examined from control and EGF-treated animals are indicated in the figure.

phosphorylation occurred in 5 min (Fig. 2, lane 3); a trace enhancement was noted after 1 min (lane 2); the extent of tyrosine phosphorylation slowly declined but was readily apparent after 45 min (lane 5); the direct injection of EGF in the liver resulted in an enhancement of tyrosine phosphorylation within 1 min (lane 8). The phosphotyrosine content of pp170 was at least 50 times greater in the EGF-treated animals than in the controls (quantitation was performed on the PhosphorImager). The subcutaneous injection of insulin resulted in only trace effects (lanes 6 and 7).

Identification of pp170 as EGFR and pp55 as an EGFR-Associated Protein. In preliminary experiments it was noted that pp170 and pp55 were coprecipitated when liver or kidney extracts from EGF-treated animals were incubated with an antiserum to the mouse liver receptor. To confirm these results, equal aliquots of liver extracts from control and EGF-treated animals were prepared and incubated with the following reagents, all of which could be expected to interact either with EGFR or with tyrosine-phosphorylated EGFR: antiserum to mouse EGFR, antiserum to human EGFR, antibodies to phosphotyrosine, EGF linked to Affi-Gel, and wheat germ lectin-Sepharose. The immune complexes were isolated using protein A-Sepharose. The proteins on the washed beads were eluted and examined by SDS/PAGE and Western blot analysis using anti-phosphotyrosine. With each adsorbent, pp170 and pp55 were coadsorbed from liver extracts of EGF-treated animals (Fig. 3). No pp170 or pp55 was detectable in liver extracts from control animals. It should be noted that when anti-phosphotyrosine (Fig. 3, lanes 5 and 6) or wheat germ lectin-Sepharose (lanes 9 and 10) was employed, additional tyrosine-phosphorylated proteins were detected in the extracts from EGF-treated animals.

We conclude that the tyrosine-phosphorylated pp170 is the mouse liver EGFR and that the tyrosine-phosphorylated pp55 is noncovalently associated with the EGFR. The adsorption of pp55 by EGF-Affi-Gel and wheat germ lectin-Sepharose also indicates that pp55 is not simply an antigenically related fragment of the EGFR that is adsorbed by the antiserum to EGFR.

To confirm these conclusions liver extracts from control and EGF-treated animals were incubated with antiserum to EGFR, and the pp170–pp55 complex was isolated using protein A-Sepharose. The complexes were eluted from the beads, separated by SDS/PAGE, and analyzed by Western blotting using both anti-phosphotyrosine antibodies and antiserum against EGFR. As expected, both pp170 and pp55 reacted with anti-phosphotyrosine antibodies in liver extracts from EGF-treated animals (Fig. 4, lane 2) but not from

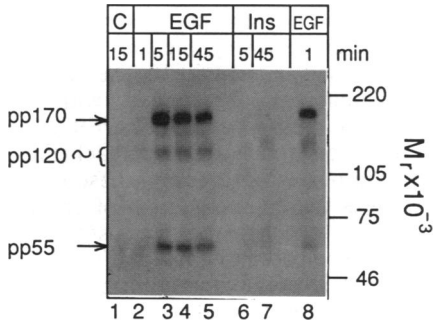

FIG. 2. Time course of EGF-dependent tyrosine phosphorylation in neonatal mouse liver. EGF (1 mg/ml), insulin (4 mg/ml), or PBS was injected at a dose of 10 μl/g of body weight. Mice were sacrificed at the indicated times following treatment. Livers were excised, and aliquots were subjected to Western blot analysis for phosphotyrosine as described in Fig. 1. Solutions were injected subcutaneously (lanes 1–7) or intrahepatically (lanes 8). Control (C), lane 1; EGF, lanes 2–5 and 8; insulin (Ins), lanes 6 and 7.

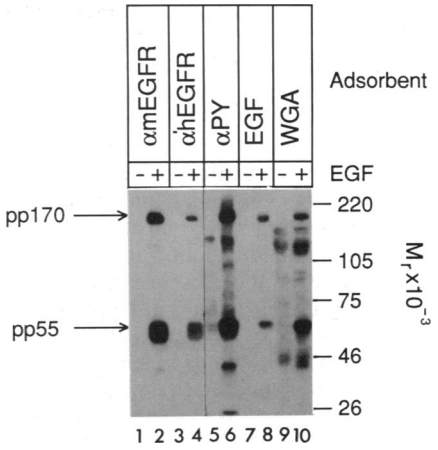

FIG. 3. Adsorption of pp170 and pp55 to matrices with affinity for the EGF receptor. EGF (1 mg/ml in PBS) or PBS was injected subcutaneously at a dose of 10 μl/g of body weight. Mice were sacrificed 5 min later, and livers were excised and processed. Aliquots (500 μl) of the clarified homogenate from control or EGF-treated animals were incubated with antiserum to mouse liver EGFR (αmEGFR) (lanes 1 and 2), antiserum to human A-431 EGFR (αhEGFR) (lanes 3 and 4), antiserum to phosphotyrosine (αPY) (lanes 5 and 6), EGF-Affi-Gel (lanes 7 and 8), or wheat germ lectin-Sepharose (lanes 9 and 10), and the mixtures were stirred for 4 hr at 4°C. Protein A-Sepharose was added 30 min prior to processing to those samples containing antisera. The adsorbed proteins were recovered, resolved by SDS/PAGE, and assayed for phosphotyrosine by Western blot analysis.

control animals (lane 1). On the other hand, pp170 was detected by antiserum against EGFR in extracts from both EGF-treated and control animals (lanes 3 and 4). No interaction of pp55 with the anti-EGFR antiserum could be detected. These results confirm the conclusions that pp170 is indeed the EGFR, that equal quantities of EGFR are recov-

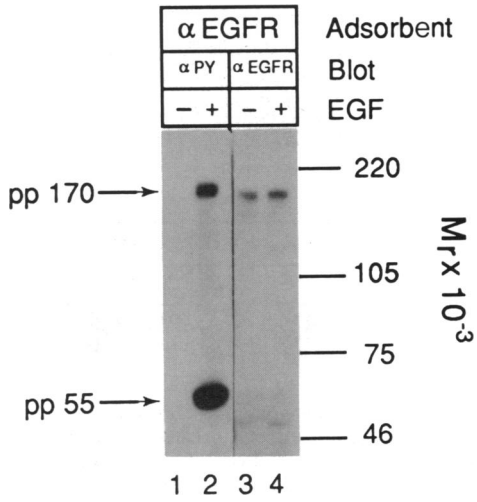

FIG. 4. Identification of pp170 as the receptor for EGF and pp55 as immunologically distinct from EGFR. EGF (1 mg/ml) or PBS was injected subcutaneously at a dosage of 10 μl/g of body weight. Mice were sacrificed after 5 min of treatment and livers were excised. Aliquots (500 μl) of clarified homogenate from control or EGF-treated animals were incubated with antiserum to mouse liver EGFR and protein A-Sepharose. The adsorbed proteins were recovered, resolved by SDS/PAGE, and transferred to nitrocellulose. Western blot analysis was used to detect proteins containing phosphotyrosine (lanes 1 and 2) or proteins that were immunologically related to mouse liver EGFR (lanes 3 and 4). Control: lanes 1 and 3; EGF: lanes 2 and 4.

ered from EGF-treated and control animals, and that pp55 is not a phosphorylated fragment of EGFR.

To determine whether pp55 is associated with EGFR in membranes, liver homogenates were prepared from control and EGF-treated animals with the standard protocol except that Triton X-100 was omitted from solubilization buffer A. The homogenates were centrifuged for 5 min at 1000 × *g* to remove debris and nuclei, and a membrane fraction was collected by centrifugation for 60 min at 150,000 × *g*. Western blot analysis of equivalent aliquots of the membrane and soluble fractions showed the presence of tyrosine-phosphorylated pp170 and pp55 in membranes prepared from EGF-treated animals but not from control animals (data not shown). pp170 was not detectable in the soluble fraction from either control or EGF-treated animals. A small amount (<20% of the total) of pp55 was seen in the soluble fraction from EGF-treated animals. The major EGF-dependent tyrosine-phosphorylated protein in the soluble fraction had an apparent molecular mass of ≈90 kDa (data not shown).

Phospho Amino Acid Analysis of ^{32}P-Labeled pp170 and pp55 Isolated from EGF-Treated and Control Mice. [^{32}P]orthophosphate (4 mCi) was injected into each of two 4-day-old mice. Two hours later EGF or PBS was injected. After 10 min, liver extracts were prepared and immunoprecipitated with anti-EGFR, and the adsorbed proteins were separated by SDS/PAGE and transferred to Immobilon-P. Both pp170 and pp55 contained ^{32}P, and both proteins were more radioactive when isolated from EGF-treated animals than when isolated from control animals (Fig. 5*A*). The phosphorylated amino acid residues in pp170 and pp55 isolated from control and EGF-treated animals were identified by limited acid hydrolysis of the appropriate bands cut from the Immobilon-P and high-voltage electrophoresis. Phosphoserine was detected in both proteins isolated from either control or EGF-treated animals (Fig. 5*B*). Phosphotyrosine also was detected in both proteins, but only in samples from the EGF-treated animals.

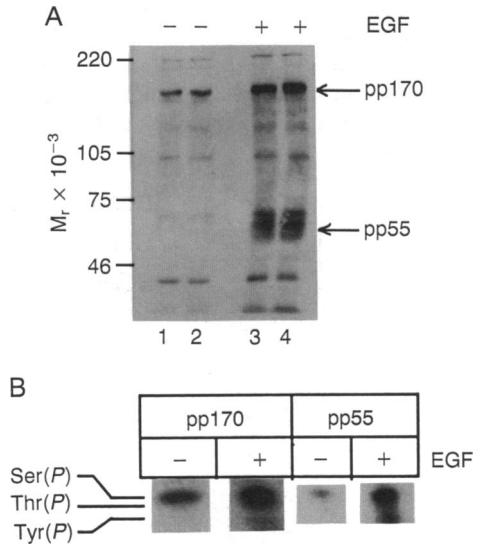

FIG. 5. Phosphorylation and phospho amino acid analysis of pp170 and pp55 isolated from EGF-treated and control ^{32}P-labeled neonatal mice. [^{32}P]Phosphate (4 mCi) was injected subcutaneously into each of two 4.5-day-old mice. Two hours later EGF or PBS was injected subcutaneously. After 10 min, liver extracts were prepared and immunoprecipitated with anti-mouse liver EGFR antiserum and protein A-Sepharose, and the adsorbed proteins were separated by SDS/PAGE and transferred to Immobilon-P. The ^{32}P-labeled phosphoproteins were detected by autoradiography (Kodak X-AR). The ^{32}P-labeled pp170 and pp55 bands were hydrolyzed and subjected to high-voltage electrophoresis. ^{32}P-labeled phospho amino acids were detected by autoradiography using a Molecular Dynamics PhosphorImager (7-day exposure). (*A*) Autoradiography of immunoprecipitates from the control (lanes 1 and 2) and EGF-treated (lanes 3 and 4) animals after SDS/PAGE. (*B*) Phospho amino acid analysis of pp170 and pp55 from the control (lanes 1 and 3) and EGF-treated (lanes 2 and 4) animals.

DISCUSSION

We were led to the use of neonatal mice for the detection of substrates phosphorylated on tyrosine in response to EGF by a number of early observations on the *in vivo* morphological and physiological effects of the administration of EGF to neonatal animals. Not only did EGF enhance epidermal growth and keratinization (leading eventually to precocious eyelid opening) (1), but it also caused a rapid induction of ornithine decarboxylase activity (within 2–4 hr) in skin, testes, liver, and kidney (13, 14). The induction of ornithine decarboxylase by EGF in the neonatal mouse was as rapid as the induction of ornithine decarboxylase in organ cultures of chicken embryo epidermis (13), indicating that other EGF-dependent responses also might be detected *in vivo*.

In the present study we demonstrated that the administration of EGF to neonatal mice increased tyrosine phosphorylation of a number of proteins in all organs examined (liver, kidney, lung, skin, testes, and brain) (Fig. 1). Enhancement of tyrosine phosphorylation was very rapid following subcutaneous administration of EGF; maximal phosphorylation in the liver was noted in 5 min and then slowly declined (Fig. 2). The relative abundance of phosphorylated tyrosine in these proteins varied depending on the organ examined (Fig. 1). The experiments reported herein were all performed with a dose of EGF of 10 μg/g of body weight. Clear responses (25–50% of maximal) were noted in the liver at a dose of 1 μg/g and could be detected at a dose of 0.1 μg/g (data not shown). Only trace effects were noted following the subcutaneous injection of insulin (Fig. 2). A response to EGF similar to that seen *in vivo* could be elicited by the direct addition of EGF to a minced liver preparation suspended in PBS (data not shown).

It is of interest that in the 0.5-day-old neonatal mouse the subcutaneous injection of EGF elicited a response in the brain within 5 min, suggesting that the blood–brain barrier is not fully developed at this time. It is not known which cells in the brain are responding; glial cells and some neurons, however, are known to possess EGFRs (1). It has been reported that EGF does not cross the blood–brain barrier in adult rats (15). Only a slight response was detected in the brains of 7-day-old animals; however, direct cranial injections of EGF into older animals again elicited a marked increase in the tyrosine phosphorylation of pp170 and pp120 (data not shown).

One of the major tyrosine-phosphorylated proteins detected in all organs following the administration of EGF (pp170) was identified as the EGFR. The identification was based on its immunological reactivity with antibodies to EGFR (antibodies both to human EGFR and to mouse EGFR) and its adsorption to EGF-Affi-Gel (Figs. 3 and 4).

An unexpected observation during these immunoprecipitation studies was the coprecipitation of one of the EGF-stimulated tyrosine-phosphorylated substrates (pp55) with EGFR in the presence of antibodies to EGFR. The association of pp55 with the receptor was confirmed by the isolation of the complex (pp170/pp55) under a variety of conditions, all of which would be expected to selectively absorb either EGFR or phosphorylated EGFR (antiserum to human or mouse EGFR, antiserum to phosphotyrosine, EGF-Affi-Gel, and wheat germ lectin-Sepharose) (Fig. 3). pp55 could not be Western blotted with antiserum to EGFR and thus is not an antigenically related fragment of the receptor. All of the pp170 and most (80%) of the phosphorylated pp55 were detected in the crude pellet fraction obtained by centrifuga-

tion of liver homogenates (prepared without detergent) from EGF-treated animals.

We have confirmed our results by immunoprecipitation of liver extracts from ^{32}P-labeled neonates with anti-EGFR antibodies. Both pp170 and pp55 contained ^{32}P and both proteins were more radioactive when isolated from EGF-treated animals than when isolated from control animals (Fig. 5*A*). Phosphotyrosine was detected in both pp170 and pp55, but only when the proteins were isolated from animals treated with EGF (Fig. 5*B*). The major phospho amino acid detected in both proteins was phosphoserine, suggesting that EGF also activates a serine kinase(s) *in vivo*. Whether threonine was phosphorylated could not be ascertained from our data.

During the course of these studies Wada *et al.* (16) reported that after intraportal injection of EGF into adult rats, isolated rat liver membranes and endosomes contained both tyrosine-phosphorylated EGFR and a tyrosine-phosphorylated 55-kDa protein. The 55-kDa protein was associated with the EGFR after immunoprecipitation with EGFR antibodies. Wada *et al.* (16) reported that the 55-kDa protein did not react with antibodies to Src or with antibodies to a 55-kDa phosphorylated cytokeratin detected in hepatocytes (17).

The EGF-dependent 55-kDa phosphorylated protein detected in a number of organs in our study appears to have characteristics similar to that detected in adult rat liver by Wada *et al.* (16). An EGF-dependent enhancement of tyrosine phosphorylation of the EGFR and an unidentified 120-kDa protein have been detected in rat hepatocyte cell suspensions by Okamoto *et al.* (18). Their figure 1 indicated that under some extraction conditions an EGF-dependent 55-kDa phosphoprotein could be detected but it was not commented upon. EGF-dependent tyrosine phosphorylation has also been reported for the rat liver-derived WB344 cell line (19) and in NIH 3T3 mouse fibroblasts overexpressing EGFR (20). Enhanced tyrosine phosphorylation of at least seven proteins, including EGFR, was noted in these studies but no association of substrates with EGFR was detected. However, four tyrosine-phosphorylated proteins, including one of 55 kDa, were found to be associated with EGFR after crosslinking with mouse B82L cells overexpressing EGFR (21). Whether these proteins and those detected in the present study are related is not known; assessment of the functional consequences of tyrosine phosphorylation of each of these substrates must await identification.

The detection of EGF-induced tyrosine phosphorylation in all organs examined suggests that physiological responses to EGF may be widespread. Identification of the specific cell types in each organ that respond to EGF may furnish a clue.

We thank Katherine Chen for technical assistance and Marlene Jayne for secretarial assistance. This work was supported by National Institutes of Health Grant HD00700 and American Cancer Society Grant RDP4D.

1. Carpenter, G. & Wahl, M. I. (1990) in *Peptide Growth Factors and their Receptors: I*, eds. Sporn, M. D. & Roberts, A. B. (Springer, New York), pp. 69–171.
2. Fisher, D. A. & Lakshamanan, J. (1990) *Endocr. Rev.* **11**, 418–442.
3. Laurence, D. J. R. & Gusterson, B. A. (1990) *Tumor Biol.* **11**, 229–261.
4. Carpenter, G. (1987) *Annu. Rev. Biochem.* **56**, 881–914.
5. Yarden, Y. & Ullrich, A. (1988) *Annu. Rev. Biochem.* **57**, 443–478.
6. Ullrich, A. & Schlessinger, J. (1990) *Cell* **61**, 203–212.
7. Hunter, T. (1989) *Curr. Opinion Cell Biol.* **1**, 1168–1181.
8. Carpenter, G. & Cohen, S. (1990) *J. Biol. Chem.* **265**, 7709–7712.
9. Carpenter, G. (1992) *FASEB J.*, in press.
10. Cohen, S., Fava, R. A. & Sawyer, S. T. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 6237–6241.
11. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
12. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
13. Stastny, M. & Cohen, S. (1970) *Biochim. Biophys. Acta* **204**, 578–589.
14. Stastny, M. & Cohen, S. (1972) *Biochim. Biophys. Acta* **261**, 177–180.
15. Nave, K. A., Probstmeier, R. & Schachner, M. (1985) *Cell Tissue Res.* **241**, 453–475.
16. Wada, I., Lai, W. H., Posner, B. L. & Bergeron, J. J. M. (1992) *J. Cell Biol.* **116**, 321–330.
17. Baribault, H. R., Blouin, R., Bourgon, L. & Marceau, N. (1989) *J. Cell Biol.* **109**, 1665–1676.
18. Okamoto, M., Karasik, A., White, M. F. & Kahn, C. R. (1990) *Biochemistry* **29**, 9489–9494.
19. McCune, B. K. & Earp, H. S. (1989) *J. Biol. Chem.* **264**, 15501–15507.
20. Fazioli, F., Bottaro, D., Minichiello, L., Auricchio, A., Wong, W., Segatto, O. & DiFiore, P. (1992) *J. Biol. Chem.* **267**, 5155–5161.
21. Cochet, C., Filhol, O., Payrastre, B., Hunter, T. & Gill, G. (1991) *J. Biol. Chem.* **266**, 637–644.