Epidermal Growth Factor Stimulated Phosphorylation of a 120-Kilodalton Endogenous Substrate Protein in Rat Hepatocytes†

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ABSTRACT: Endogenous substrates of the EGF receptor have been described in transformed cells; however, little is known about substrates in normal tissue. To characterize epidermal growth factor (EGF) receptor phosphorylation and search for endogenous substrates in normal rat hepatocytes, cells were labeled with [32P]orthophosphate, and phosphotyrosine-containing proteins were sought by using a high-affinity, specific anti-phosphotyrosine antibody. Exposure of [32P]-labeled freshly isolated hepatocytes to 1 μg/mL EGF caused phosphorylation of several proteins of M, 185K, 160K, and 120K. The 185- and 160-kDa proteins (pp185 and pp160) were identified as the intact and proteolyzed forms of the EGF receptor by virtue of their immunoprecipitation with anti-EGF receptor antibody. This antibody failed to recognize the 120-kDa phosphoprotein (pp120). The phosphopeptide map derived from pp120 was by trypsinization and HPLC separation different from that of pp185, further indicating that pp20 is different from the EGF receptor. This pp120 was also immunologically distinct from the pp120 substrate of the insulin receptor kinase and from ATP-citrate lyase. Phosphoamino acid analysis revealed pp120 to be phosphorylated on both tyrosine and serine residues. Autophosphorylation of EGF receptor and phosphorylation of pp120 were almost maximal within 1 min of EGF stimulation. The dose–response curves for phosphorylation of the EGF receptor and pp120 were identical (ED50 = 30 ng/mL) and were superimposable with the fractional occupancy of the EGF receptor. In A431 cells, a transformed cell line whose growth is inhibited by EGF, EGF produced a decrease in pp120 phosphorylation. These data suggest that pp120 is an endogenous substrate for the EGF receptor in hepatocytes whose phosphorylation may be closely related to EGF stimulation of cell growth.

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The regulatory action of epidermal growth factor (EGF) on cellular metabolism and growth is initiated by the binding of the hormone to its receptors on the surface of cells. The EGF receptor is a transmembrane glycoprotein with intrinsic protein tyrosine kinase activity (Ullrich et al., 1984; Weber et al., 1984; Hunter & Cooper, 1985). Upon binding to the hormone, this kinase is activated and causes tyrosine phosphorylation of the receptor itself and of other intracellular proteins. One model for transmembrane hormone signal transduction suggests that these proteins in their phosphorylated form serve as messengers for the EGF signal. Support for this hypothesis is found in recent studies using cells transfected with a mutant EGF receptor lacking the kinase activity which failed to induce any of the many diverse biochemical effects of EGF (Chen et al., 1984; Glenny et al., 1988). A variety of purified exogenous proteins (House et al., 1984; Ghosh-Dastidir et al., 1984), proteins in membranes and cell homogenates (Gould et al., 1986; Cassel et al., 1983), and synthetic peptides (Hunter & Cooper, 1981) have been identified to serve as substrates for the EGF receptor kinase in vitro. In A431 cells, a human epidermoid carcinoma cell line, several proteins have been shown to undergo an increase in tyrosine phosphorylation upon EGF stimulation (Hunter & Cooper, 1981; Gould et al., 1986; Fava & Cohen, 1984; De et al., 1986). Two of these proteins have been identified as lipocortin 1 (Sawyer & Cohen, 1985; Pepinsky & Sinclair, 1986) and a 145-kDa isoenzyme of phospholipase C (Wahl et al., 1988, 1989). Although it is not clear if the activity of these proteins is altered, their phosphorylation has been suggested to play a role in EGF signal transmission. On the other hand, only a few studies have attempted to identify endogenous substrates for the EGF receptor in nontransformed cells (Richman et al., 1976; Lin et al., 1984).

Normal hepatocytes possess a relatively large number of EGF receptors (more than 300 000 per cell) and have been reported to respond to EGF with a stimulation of growth (Giungi et al., 1985; McGowan et al., 1981; Earp & O’Keefe, 1981). In this study, we have utilized anti-phosphotyrosine antibodies to study EGF receptor phosphorylation and identify possible endogenous substrates in isolated rat hepatocytes. We find that hepatocytes possess a protein of M, 120 000 which is rapidly phosphorylated on tyrosine residues in an EGF-dependent manner. This protein has characteristics of an endogenous substrate for the EGF receptor which may be linked to EGF effect on cellular growth.

Materials and Methods

Materials. Sprague-Dawley rats were obtained from Charles River Laboratory, collagenase (type IV) was from Cooper Biomedical, [32P]orthophosphate and Triton X-100 were from New England Nuclear, phosphoamino acids were from Sigma, porcine insulin was from Elanco, reagents for SDS-PAGE were from Bio-Rad, and receptor-grade EGF was from Collaborative Research. Pansorbin was purchased from Calbiochem and protein A-Sepharose from Pharmacia.

†Abbreviations: EGF, epidermal growth factor; kDa, kilodalton(s); M, apparent molecular weight; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; DTT, dithiothreitol.
RPML 1640 tissue culture medium was from GIBCO. Anti-EGF receptor antibody (RK2) prepared to a synthetic peptide with the sequence of an intracellular domain of the EGF receptor was a generous gift of Dr. J. Schlessinger (Kris et al., 1985). Polyclonal anti-phosphotyrosine antibody was prepared in rabbits and purified by affinity chromatography as previously described (Pang et al., 1985). Antiserum to ATP-citrate lyase was a gift of Dr. J. Avruch (Massachusetts General Hospital, Boston, MA).

Isolation and $^{32}$P Labeling of Hepatocytes. Hepatocytes were isolated by using a modification of the method of Berry and Friend (Okamoto et al., 1982) from male Sprague-Dawley rats weighing 160–220 g fed ad libitum. The cells were washed with 137 mM NaCl supplemented with 2.7 mM KCl and 20 mM HEPES, pH 7.4, and resuspended in phosphate-free RPMI 1640 to give a final concentration of 2 x $10^6$ cells/mL. Labeling of the hepatocytes with $[^{32}$P]orthophosphate was achieved by incubating aliquots (0.5 mL) of cell suspension for 90 min with $[^{32}$P]orthophosphate (1 mCi/point) at 37 °C.

Phosphorylation of EGF Receptor and Endogenous Substrates. After $^{32}$P labeling, cells were stimulated with EGF. The reaction was stopped by using one of two methods (White et al., 1985). For Triton X-100 extracts, the reaction was stopped by adding 0.5 mL of ice-cold stopping solution to give the following final concentrations: 50 mM HEPES, pH 7.4; 1% Triton X-100; 10 mM sodium pyrophosphate; 100 mM sodium fluoride; 10 mM EDTA; 2 mM sodium vanadate; 2 mM phenylmethanesulfonyl fluoride; 0.1 mg/mL aprotinin. The mixture was vigorously vortexed, cooled with dry ice/methanol until minimal ice was detected in the bottom, vortexed again, and kept on ice for 30 min. For SDS extraction, 0.5 mL of boiling stopping buffer was added to give the same concentrations as described above except that 1% SDS replaced the 1% Triton, and PMSF and aprotilin were omitted. After the sample was boiled for 15 min, it was cooled on ice for 60 min. After both extraction procedures, the samples were centrifuged at 200000g for 45 min, and supernatant was used for immunoprecipitation.

Immunoprecipitation with Anti-Phosphotyrosine Antibody and Anti-EGF Receptor Antibody. Immunoprecipitation with anti-phosphotyrosine antibody was performed at a dilution of 1:100 with pansorbin as previously reported (White et al., 1985). Immunoprecipitation with anti-EGF receptor antibody was performed in similar manner using a 1:200 dilution of antibody (Kris et al., 1985). The proteins were then solubilized in Laemmli buffer with 100 mM dithiothreitol and separated in 7.5% polyacrylamide gels by electrophoresis (Laemmli, 1970). The gels were stained with Coomassie blue in 50% trichloroacetic acid, destained in 7% acetic acid, dried, and autoradiographed with Kodak X-Omat film. Molecular weights of the labeled proteins were calculated by using the following protein standards: myosin, $M_r = 200K$; $\beta$-galactosidase, $M_r = 116K$; phosphorylase b, $M_r = 94K$; bovine serum albumin, $M_r = 66K$; ovalbumin, $M_r = 45K$. The incorporation of $^{32}$P into protein bands was quantitated by scanning densitometry of the film or by counting the corresponding bands of the gel in a scintillation counter.

Phosphoamino Acid Analysis. Tryptic phosphopeptides were obtained from the protein bands in polyacrylamide gel fragments as previously described (Kasuga et al., 1984; Haring et al., 1984). Gel fragments containing phosphorylated proteins were excised, washed for 12 h at 37 °C in 20% methanol, dried at 80 °C for 2 h, and digested with 2 mL of 50 mM NH$_4$HCO$_3$ containing 100 $\mu$g of trypsin (pH 8.0). After a 6-h incubation at 37 °C, another 100 $\mu$g of trypsin was added, and the enzymatic digestion was continued for an additional 16 h. The supernatant solution was lyophilized, and the phosphopeptides were dissolved in 100 $\mu$L of 6 N HCl and hydrolyzed for 2 h at 110 °C. The phosphoamino acids were separated by high-voltage electrophoresis on thin-layer plates (Avicel, Analtech, Newark, DE: 250 $\mu$m) using a solution of H$_2$O/acetic acid/pyridine (89:10:1). Unlabeled phosphoserine, phosphothreonine, and phosphotyrosine standards were added to all samples and identified by reaction with ninhydrin, and the radioactivity was located by autoradiography.

HPLC Separation of the Tryptic Phosphopeptides. The phosphopeptides were eluted from the gel fragment (nearly 95%) as described above and were separated with a Waters high-performance liquid chromatography system equipped with a wide-pore C$_18$ column (Bio-Rad, RP-318). Phosphopeptides were applied to the column which was washed with 5% acetonitrile and eluted with an acetonitrile gradient increasing linearly to 75% during 105 min.

RESULTS AND DISCUSSION

Intact rat hepatocytes were exposed to 1 $\mu$g/mL EGF for 10 min, and their proteins were extracted with 1% Triton or 1% SDS as described under Materials and Methods. Tyrosine-phosphorylated proteins were precipitated with anti-phosphotyrosine antibody and analyzed by SDS–PAGE under reducing conditions (Figure 1). No phosphorytosine-containing proteins were seen in the baseline state, though longer exposure of the autoradiograms revealed slight phosphorylation of proteins at $M_r$ 175K and 120K in the baseline state. Exposure to EGF consistently caused phosphorylation of three proteins: a doublet of $M_r$ 185K and 190K and an additional band at $M_r$ 120K. In some experiments, weakly phosphorylated bands of $M_r <100K$ were also phosphorylated. Recovery of the 185-, 190-, and 120-kDa bands was much better when boiling SDS, rather than Triton X-100, was used to terminate the phos-
phorylation reaction and to extract the proteins. This is most probably due to complete arrest of phosphatase and protease activities in the extracts boiled in SDS.

Two forms of EGF receptor have been identified in previous studies (Cohen et al., 1980, 1982). These have been assigned molecular weights of 170000 and 150000. In our experiments using hepatocytes, anti-EGF receptor antibodies precipitated both proteins of M₁ 185K and 160K, identifying them as the EGF receptor. Cohen et al. have shown that both forms that bind EGF are immunoprecipitated by anti-EGF receptor antibody and are phosphorylated in the presence of ATP in an EGF-dependent manner (Cohen et al., 1980). The lower molecular weight form appears to be derived from the larger form by the action of a Ca²⁺-sensitive neutral protease which removes a 20-kDa fragment from the carboxyl terminal (Cohen et al., 1982). This change in form of the EGF receptor increases its kinase activity against certain exogenous substrates and reduces autophosphorylation activity. In our experiments, labeling of the 160-kDa form was greater than that of the 185-kDa form under most conditions. Both forms were observed when the receptor was extracted with boiling SDS and in the presence of protease inhibitors. Whether this limited proteolysis of the EGF receptor is important for signal transmission is unknown, but there was no change in the ratio of the 185- and 160-kDa forms from 1 to 30 min of EGF stimulation.

Preliminary identification of pp120 was attempted by immunoprecipitation with antisera raised against the EGF receptor and against several known phosphoproteins with a similar molecular weight that exist in liver. Anti-EGF receptor antibodies that precipitated both pp160 and pp185 failed to recognize the 120-kDa proteins (data not shown). A substrate of both the insulin and EGF receptor kinase of M₁ 120K has been reported in wheat germ agglutinin and purified Triton extracts of rat liver membranes (Rees-Jones & Taylor, 1985; Phillips et al., 1987; Fanciulli et al., 1989; Perrotti et al., 1987). This protein that serves as a substrate for the insulin and IGF-1 receptor kinases in a hepatoma cell line has been recently identified as an integral membrane glycoprotein of the bile canaliculus (Margolis et al., 1988). The pp120 present in Triton X-100 extracts of hepatocytes in the current study was not immunoprecipitated by an antibody to this previously described pp120 (data not shown). Furthermore, the pp120 in the current study does not bind to WGA (P. L. Rothenberg and C. R. Kahn, unpublished results), suggesting that these two ~120-kDa proteins are distinct. Our pp120 was also not precipitated by an antibody to ATP-citrate lyase, a protein which has a similar molecular weight (data not shown).

As the EGF receptor can yield a ~125-kDa degradative fragment after limited proteolytic cleavage (Chinkers & Brugge, 1984), it was imperative to establish that pp120 observed in this study is not derived from the EGF receptor. Much evidence exists to this effect. First, the protein was not immunoprecipitated by an antibody against the intracellular domain of the EGF receptor, indicating that it lacks the epitope recognized by the antibody. Second, no accumulation of pp120 was seen with increased time of stimulation as might be expected for a degradation product of the receptor. Third, the 125-kDa protein derived from the EGF receptor limited tryptic cleavage, lacks the major tyrosine sites of phosphorylation (Chinkers & Brugge, 1984), and is not expected to be precipitated with an anti-phosphotyrosine antibody. The most convincing evidence that pp120 is distinct from the EGF receptor is the tryptic phosphopeptide maps of both proteins. After EGF stimulation, the EGF receptor and pp120 were purified by immunoprecipitation with the anti-phosphotyrosine antibody and SDS-PAGE. The phosphopeptides obtained after exhaustive trypsin digestion of the β-subunit were separated on an RP-318 reverse-phase column (Figure 2). When an acetonitrile gradient increasing linearly from 0 to 75% over 105 min was used, most of the radioactivity in pp120 was detected in peptides eluted between 55 and 62 min. This was distinct from the peptide map of the EGF receptor which yielded peptides at 18, 20, 60, and 75 min. The difference in the peptide maps strongly implies that pp120 and pp185 are different proteins. In a separate analysis using an acetonitrile gradient increasing linearly from 0 to 25% over 85 min to analyze tryptic peptides derived from pp120, no phosphopeptides were eluted up to 25% acetonitrile, while pp185 gave a profile identical with that previously described by us for the phosphorylated EGF receptor in A431 cells (Carpentier et al., 1987).

Further characterization of EGF-stimulated phosphorylation of pp120 was conducted including the time course, dose dependence, and exact phosphoamino acid content. Labeled rat hepatocytes stimulated by EGF for 1-30 min were extracted with SDS, precipitated with anti-phosphotyrosine antibody, and analyzed by SDS-PAGE (Figure 3). Within 1 min after EGF stimulation, phosphorylation of the EGF receptor and pp120 was detected and near-maximal. There was a slight further increase in phosphorylation for the next 20 min and then a slight decrease in phosphorylation by 30 min. The decrease in phosphorylation of pp120 with time appeared to be somewhat greater than that of the EGF receptor, suggesting a faster rate of turnover of phosphotyrosine on this protein. The autoradiogram chosen was a product of longer exposure.
as described under Materials and Methods, and the phosphorylated and separated by SDS-PAGE with a patocyte proteins. Isolated rat hepatocytes were labeled with proteins were solubilized in precipitated with anti-phosphotyrosine antibody, reduced with DTT, and separated by SDS–PAGE with a 7.5% resolving gel, and the gel was subjected to autoradiography.

FIGURE 3: Time course of EGF-stimulated phosphorylation of hepatocyte proteins. Isolated rat hepatocytes were labeled with [32P]orthophosphate for 90 min, and the cells were treated with EGF (1 μM/mL) for the indicated time intervals. The reaction was stopped as described under Materials and Methods, and the phosphorylated proteins were solubilized in 1% SDS. The proteins were immunoprecipitated with anti-phosphotyrosine antibody, reduced with DTT, and separated by SDS–PAGE with a 7.5% resolving gel, and the gel was subjected to autoradiography.

FIGURE 4: Dose–response curve for EGF stimulation of [32P]incorporation into endogenous hepatocyte proteins. After incubation of intact rat hepatocytes with [32P]orthophosphate, the cells were treated with EGF concentrations from 0 to 1000 ng/mL. The phosphotyrosine-containing proteins were extracted and analyzed by SDS–PAGE as in Figure 2. The radioactivity in pp185, pp160, and pp120 was determined by cutting from the gel and counting each fragment with a scintillation counter. The cpm were then calculated as the percent of maximal increase above basal for each phosphorylation.

To illustrate the ratio between basal and EGF-stimulated phosphorylation of these bands. As neither band was seen on Coomassie blue or silver stains of extracts from 2 × 10⁶ hepatocytes, no exact stoichiometry calculations of these proteins are possible.

Stimulation by concentrations as low as 10 ng/mL EGF for 1 min led to a detectable increase in phosphorylation of the EGF receptor and pp120. Labeling of all three proteins increased further with increasing concentrations of EGF. To quantitate these data, the labeled bands were excised and counted directly in a scintillation counter. When expressed as a percent of maximal response above basal, the dose–response curves for pp185, pp160, and pp120 were almost identical (Figure 4). Half-maximal stimulation occurred at an EGF concentration of about 30 ng/mL and maximal stimulation at 1 μg/mL. When the EGF dose–response curves for EGF receptor and 120-kDa phosphorylated were compared with the percent of fractional occupancy of the EGF receptor derived from the data previously published by us (Okamoto et al., 1988), the curves were virtually superimposable, suggesting a close relationship between EGF binding to its receptor, EGF receptor autophosphorylation, and the phosphorylation of pp120.

Figure 5 shows phosphoamino acid analysis of EGF-stimulated pp120. Under basal conditions using anti-phosphotyrosine antibody precipitation, phosphorylation of the EGF receptor and phosphorylation of pp120 were almost undetectable, indicating that these proteins were phosphorylated on tyrosine residues only in response to EGF stimulation. This is further supported by the phosphoamino acid analysis, which revealed phosphotyrosine as a significant constituent of pp120 after EGF stimulation. In addition, there was a major increase in phosphoserine in pp120. Whether this increase in phosphoserine arises from de novo phosphorylation or simply improved recovery by the phosphotyrosine antibody following tyrosine phosphorylation is unknown.

Since most studies of EGF-related phosphorylation have utilized transformed cells, and in particular A431 cells, we compared EGF-stimulated phosphorylation of endogenous proteins in two transformed cell lines [Fao cells, a well-differentiated rat hepatoma cell line (White et al., 1985), and A431 cells, a human epidermoid carcinoma (Carpentier et al., 1987)] to our findings in hepatocytes. The latter cells are well-known as one of the cells with the highest concentration of EGF receptors; however, in these cells, EGF suppresses cell growth rather than stimulates it (Cohen et al., 1980; Carpentier et al., 1987). Fao cells, on the other hand, do not have EGF receptors and have no known EGF responses either positive or negative.

Not surprisingly, when Fao cells were subjected to EGF stimulation, no EGF receptor was detected using anti-phosphotyrosine antibody, and no increase in phosphorylation of pp120 was observed (Figure 6, lanes a and c). In contrast, in A431 cells, EGF receptor phosphorylation was markedly stimulated by EGF, as was the phosphorylation of proteins of M₆, 105K, 65K, and 46K (Figure 6, lanes d and f). Interestingly, in A431 cells, the phosphorylation of pp120 was decreased in response to EGF stimulation. For comparison, we show insulin-stimulated phosphorylation of proteins of M₆, 95K and ~175K, corresponding to the β-subunit of the insulin receptor and its endogenous substrate in both Fao and A431 cells (Figure 6, lanes b and e). The EGF-induced decrease in phosphorylation of pp120 in A431 cells is noteworthy as in these cells EGF inhibits growth. Assuming that the pp120 in A431 is identical with that found in hepatocytes, these data may be expressing a relationship between EGF's ability to stimulate cell growth and the phosphorylation of pp120. The reason for the high basal level of phosphoryrosine of pp120 in Fao cells and A431 cells as compared with freshly isolated hepatocytes is still unknown.
Then the cells were stimulated with each group, the first lane is basal, the second lane is after stimulation A431 cells and Fa0 cells. Confluent monolayers of cells grown in substrate for the EGF receptor kinase in hepatocytes (Karasik et al., 1988; Karasik et al., 1989) phosphorylated in response to tumor promoter stimulation and the third lane is after EGF stimulation. An autoradiogram of the dried gel is shown. The first group of three lanes are from Fao cells; the second group are from A431 cells. In each group, the first lane is basal, the second lane is after stimulation with insulin, and the third lane is after EGF stimulation.

The best characterized endogenous substrate for the EGF receptor tyrosine kinase is lipocortin 1 (Fava & Cohen, 1984; Huang et al., 1986; Pepinsky & Sinclair, 1986). Lipocortin 1 has been found to be phosphorylated in response to EGF in A431 cells (Fava & Cohen, 1984; Sawyer & Cohen, 1985), in HL-60 cells (Williams et al., 1988), and in human placenta (Valentine-Braun et al., 1987). Lipocortin is also a weak substrate for the EGF receptor kinase in hepatocytes (Karasik et al., 1988; Karasik & Kahn, 1988), and a Triton-extractable form of lipocortin I has been identified in T51B rat liver cells which is phosphorylated in response to tumor promoter (Compos-Gonzalez, 1989). Lipocortin 1 is unlikely to serve as a second messenger in EGF signal transmission since its phosphorylation is rather delayed, being maximal at 60 min after EGF binding. By contrast, pp120 is rapidly phosphorylated.

The exact nature of pp120 remains unknown. The pp120 identified in this study is distinct from that identified in rat hepatoma cells by Fanciulli et al. (1989). A 120-kDa cell adhesion molecule which encodes a phosphotyrosinolinked, PI-specific phospholipase C has been identified in skeletal muscle (Barton et al., 1988). In view of the findings that other isoforms of phospholipase may be substrates of the EGF receptor (Wahl et al., 1989; Margolis et al., 1989), one might speculate that the hepatic pp120 is a phospholipase or cell adhesion molecule. A 120-kDa GTPase-activating protein has also been identified as a substrate of the PDGF receptor tyrosine kinase (Molloy et al., 1989). In recent studies (P. L. Rothenberg and C. R. Kahn, unpublished results) using perfused intact rat liver, we have observed an EGF-stimulated phosphorylation of a pp120 with similar characteristics to those present in the isolated hepatocytes. We have succeeded in the purification of this pp120 and have limited a peptide sequence which indicates that it is distinct from the known phospholipases in GTP-activating proteins and is unique in the data bank. Taken together, these data indicate that the pp120 of liver may be a new protein that is involved in EGF signal transduction in normal tissues.

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