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EPIDERMAL GROWTH FACTOR-STIMULATED PHOSPHORYLATION OF TYROSINE RESIDUES ON A 120 000 DALTON PROTEIN IN MOUSE LIVER PLASMA MEMBRANE SUBFRACTIONS

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SUMMARY

The addition of Epidermal growth factor (EGF) to mouse liver sinusoidal plasma membrane subfractions, isolated from the blood face of hepatocytes, resulted in a two to threefold increase of ^{32}P incorporation from [γ-^{32}P]ATP into a 120 000 dalton protein component. The EGF-stimulation of the phosphorylation of this component was also evidenced in plasma membranes derived from the lateral and biliary faces of hepatocytes. A major phosphoamino acid residue after EGF stimulation was identified as tyrosine. An EGF-enhancement of the phosphorylation of threonine and serine residues also appears to be detectable.

INTRODUCTION

The specific binding of Epidermal growth factor (EGF) to plasma membrane receptors in target cells elicits various events including mitogenesis (1), regulation of cellular differentiation (2) and inhibition of gastric acid secretion (3).

An EGF-binding protein, which is presumably the receptor, has been partially purified from human carcinoma A-431 (4) and placenta (5) membranes. It appears to be a glycoprotein of molecular weight 150-170,000 (4) or 160-180,000 (5).

A protein kinase activity associated with the EGF receptor is rapidly stimulated in the presence of EGF (6,7). Recently, it has been shown in tumoral A-431 cell membranes that this protein kinase phosphorylates tyrosine residues (8). Activation of a tyrosine protein kinase activity may be one of the initial biochemical signals generated after interaction of EGF with its receptor (6, 7). The unusual modification of proteins by phosphorylation of tyrosine residues is a rare event which also occurs with the transforming gene product of RNA oncogenic viruses and is believed to be related to the transformation process (9-11).

Though a number of membrane proteins are phosphorylated after EGF stimulation, only one or two components are primarily affected in the 150-180,000

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dalton range, as observed for membranes isolated from A-431 cells, fibroblasts, placenta (7) and kidney cells (12). The EGF receptor thus appears to be a prominent membrane protein substrate for an EGF-stimulated tyrosine protein kinase activity in vitro, at least in A-431 cells.

The significance of the EGF-enhanced phosphorylation of membrane proteins is not established. As it is the case for cAMP-dependent protein kinases and oncogenic RNA virus-induced kinases, activation of protein kinase(s) by EGF may explain the pleiotropic effect required for the mitogenic process. The EGF-activated phosphorylation of the EGF receptor may be related to the mechanism of internalization of EGF-receptor complexes and to down-regulation of the number of EGF receptors. Therefore it is of interest to study the phosphorylation response to EGF of plasma membranes from liver which show much lower levels of receptors than A-431 cells or placenta (13,14). In addition, the liver EGF receptor has been tentatively characterized as a glycoprotein of about 100 000 daltons (15), in contradistinction with the 150-180 K values reported for A-431 cells (4) and placenta (5). The regional devolution of a number of plasma membrane functions on hepatocytes and interdomain dynamics in a functional epithelium have led us to study the EGF-stimulated phosphorylation reaction in membrane subfractions derived from the three major anatomical and functional surface domains in the liver : blood sinusoidal, contiguous and bile canalicular (16).

MATERIALS AND METHODS

Subcellular fractionation :

Sinusoidal plasma membrane subfractions were prepared from the livers of unfed Swiss male mice (30 ± 5 g), essentially as described by Poupon and Evans (17). The white band of sinusoidal plasma membranes was collected at the 8.5-38.8 (w/v) interface and centrifuged at 100 000 x g_{av} for 2 h after dilution with three volumes of 0.25 M sucrose, 0.005 M Tris-HCl, pH 7.5. The resulting pellets were stored in aliquots at -80°C until used. Contiguous and canalicular plasma membrane subfractions were isolated as described in (18).

EGF :

EGF was purchased from Collaborative Research, Inc. The biological activity of EGF was checked by its ability to promote early opening of the eyelids in the Swiss mouse newborn, as described by Cohen (19).

Inactivation of EGF was obtained by iodoacetamidation of reduced samples, according to Dwyer and Blobel (20). When mentioned, eventual contaminant protein kinase activities in commercial EGF preparations were inactivated by heating at 100°C for 3 min.

Standard membrane phosphorylation reaction :

Membrane subfractions were washed prior to phosphorylation assays with 0.15 M NaCl, 0.010 M Tris-HCl, pH 7.5, according to Kremmer et al (21). Endogenous phosphorylation assay was performed in 50 µl of reaction mixture containing : membranes (42.5 µg of protein, determined according to (22)); Tris-HCl buffer (50 mM, pH 7.2), $MnCl_2$ (1 mM); [γ-^{32}P] ATP (5.10^{-2} mM ; 6 to 10 x 10^5 cpm); EGF (2.2 x 10^{-4} mM); bovine serum albumin (10 µg, to prevent non spe-

cific adsorption). The reaction tubes were preincubated on ice for 10 min in the presence or absence of EGF. The reaction was initiated by the addition of labeled ATP to the mixture at 0°C and incubation was continued for 15 min at 0°C, according to Carpenter et al (6). The reaction was terminated by the addition of 18 µl of concentrated (x 4) dissolving sample buffer for SDS gel electrophoresis (23), containing SDS and Triton X-100 both at final concentrations of 2 %.

Analytical procedures :

SDS-polyacrylamide slab gel electrophoresis

Electrophoresis was carried out in 7.5, 12 or 15 % acrylamide, 0.1 % bisacrylamide slab gels in 0.1 % SDS, using the discontinuous buffer system of Laemmli and Favre (23). Dissolved samples were incubated at room temperature for 40 min (24) prior to electrophoresis. Molecular weight standards used for calibration of the gels were : β-galactosidase (130 000), phosphorylase a (94 000), bovine serum albumin (68 000), ovalbumin (43 000), carbonic anhydrase (30 000) and soybean trypsin inhibitor (20 100). Unless otherwise indicated, gels were processed through fixing, staining, destaining and drying, then submitted to autoradiography, as described previously (25).

Identification of phosphotyrosine-containing polypeptide bands after polyacrylamide slab gel electrophoresis

Stained gels were incubated for 2 h in 1 M KOH at 55°C according to Hunter and Sefton (9). They were then dried and submitted to autoradiography as in (25).

Identification of the phosphoamino acids after partial acid hydrolysis

The phosphoamino acid content of the ^{32}P-labeled membranes (120 µg of protein) was determined as described by Hunter and Sefton (9) and Ushiro and Cohen (8). Partial acid hydrolysis was performed in 6N HCl at 110°C for 2 h under N_2 atmosphere. The hydrolysate was analyzed on cellulose thin-layer sheets (Kodak) by electrophoresis at pH 3.5 (1 kV; 70 min) in acetic acid/pyridine/H_2O (10:1:189, v/v). The markers (phosphotyrosine, phosphothreonine and phosphoserine) were detected by staining with ninhydrin.

RESULTS

The incubation of mouse liver blood sinusoidal plasma membrane subfractions with [γ-^{32}P]ATP resulted in the incorporation of radioactivity into proteins, the pattern of which was revealed by autoradiography of the Coomassie blue-stained and dried SDS gel electrophoregrams (Fig. 1A). A number of phosphorylated polypeptides appeared in the absence or presence of 2.2×10^{-7} M EGF (Fig. 1A, lanes b and c), without apparent correlation between the intensity of protein staining (lane a) and the intensity of protein phosphorylation (lane c - 15 %). The membranes labeled in the presence of EGF showed essentially one protein which incorporated substantially different amounts of ^{32}P relative to the controls. This protein was assigned a molecular weight of 120 000 (lanes c). It is a minor membrane component on the basis of Coomassie blue staining (lane a) which was weakly phosphorylated in the basal state (lanes b).

The relative level of ^{32}P incorporation into the 120 000 dalton protein component both in the presence or absence of EGF can be compared from the

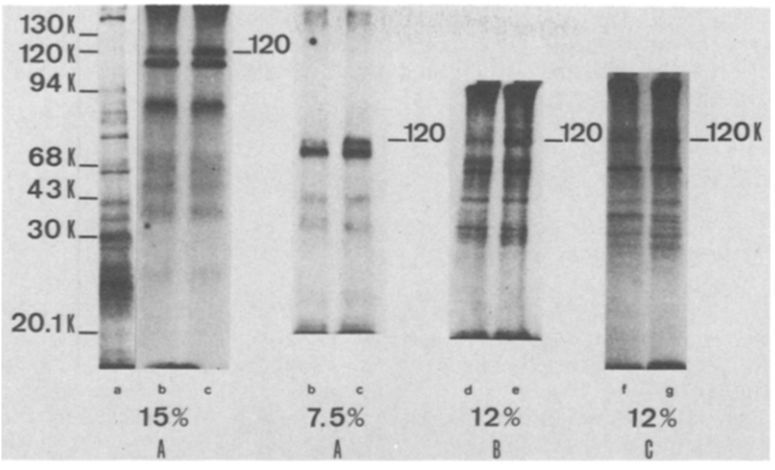

Fig. 1 : EGF-dependent phosphorylation of mouse liver plasma membrane subfractions. A : sinusoidal membranes; B : contiguous membranes; C : canalicular membranes. The phosphorylated samples were subjected to SDS gel electrophoresis, Coomassie blue staining (a) and autoradiography (b to g). The phosphorylation assays were performed in the absence (b, d, f) and presence (c, e, g) of 2.2×10^{-7} M EGF. Electrophoresis was carried out in 7.5, 12 or 15 % acrylamide slab gels. 42 µg of protein were applied to each lane.

densitometer scans of autoradiographs of the gels. The ^{32}P incorporation in the presence of EGF was stimulated by a factor of 2 to 3.

The EGF-stimulation of the phosphorylation of the 120 K component was also observed in plasma membrane subfractions isolated from the lateral (Fig. 1B, lane e) and biliary (Fig. 1C, lane g) faces of liver cells.

The linkage of the phosphate incorporated into membrane proteins was studied for its stability to acid and alcali treatments. Incubation of the stained gels in 50 % perchloric acid for 15 min at 90°C showed that the linkage of the phosphate incorporated into the 120 K protein after EGF stimulation was relatively stable to acid treatment (not shown). On the other hand, the alkali stability of phosphoproteins may be an indicator of the presence of phosphotyrosine (9). The 120 K protein from blood sinusoidal membranes exposed to EGF contains significant amounts of phosphate after incubation of the 15 % acrylamide slab gels in 1 M KOH at 55°C for 2 h (Fig. 2A, lane b). This suggested that during EGF stimulation tyrosine was a phosphate acceptor, but did not exclude threonine residues since Cooper and Hunter have shown that the results of alkali treatment depend upon the nature of phosphoproteins (11). In addition to phosphotyrosine-containing proteins, phosphoproteins which contain phosphothreonine, and in few cases phosphoserine, may show alkali-resistance. The alkali stability of the 120 K protein was also evidenced in contiguous and

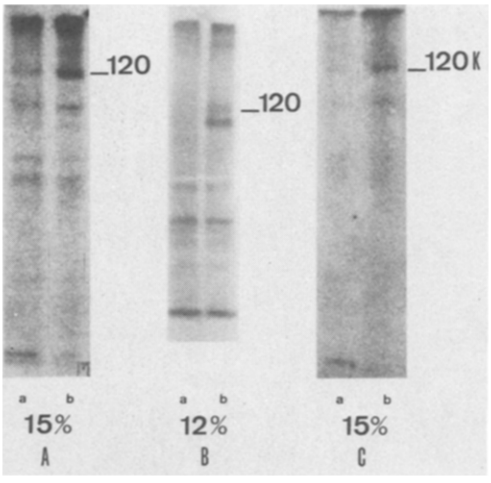

Fig. 2 : Effect of alkali treatment on the phosphoprotein pattern of plasma membrane subfractions phosphorylated in the absence (a) and presence (b) of 2.2 x 10^{-7} M EGF. The 12 or 15 % acrylamide slab gels were incubated in 1 M KOH for 2 h at 55°C (9) and submitted to autoradiography. A : sinusoidal membranes; B : contiguous membranes; C : canalicular membranes. 42 µg of protein were applied to each lane.

canalicular membrane subfractions after KOH treatment of the slab gels (Fig. 2B, lane b; Fig. 2C, lane b).

Sinusoidal membranes phosphorylated in the presence and absence of EGF were subjected to partial acid hydrolysis and electrophoretic separation at pH 3.5 of the acid-stable products. The results of autoradiography are shown in Fig. 3A. In addition to labeled peptides (spots 1) and inorganic phosphate (spot 5), the phosphorylated components comigrated with phosphotyrosine (spot 2), phosphothreonine (spot 3) and phosphoserine (spot 4). EGF severalfold stimulated the phosphorylation of tyrosine residues, in agreement with the results of Ushiro and Cohen on A-431 cell membranes in vitro (8). In contrast with these peculiar membranes, phosphothreonine also appears to be enhanced while serine was only weakly phosphorylated (Fig. 3A). From preliminary experiments on contiguous plasma membrane subfractions, increase in the content of phosphotyrosine, phosphoserine and to less extent phosphothreonine can also be detectable in such membranes after EGF stimulation (Fig. 3B).

DISCUSSION

By gel electrophoresis and autoradiographic methods, we present evidence that EGF stimulates primarily in vitro the phosphorylation of a 120 000 dalton protein in mouse liver sinusoidal plasma membranes. The EGF-stimulated phosphorylation of the 120 K protein was abolished by inactivation (iodoacetamida-

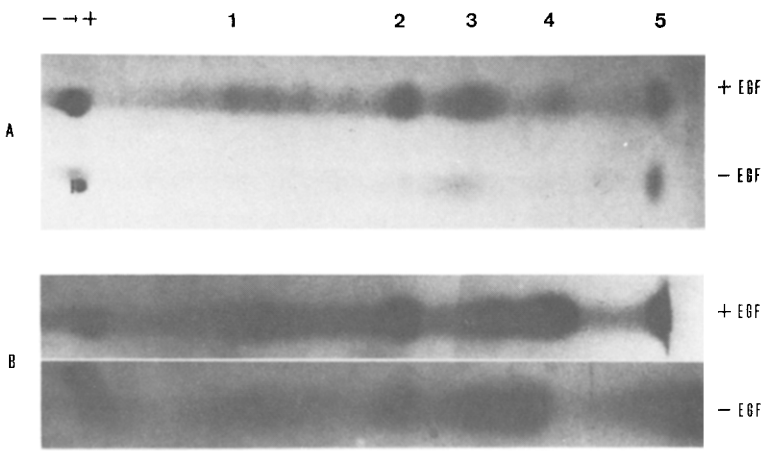

Fig. 3 : Electrophoretic separation and autoradiography of phosphoamino acids derived from sinusoidal (A) and contiguous (B) plasma membranes. Electrophoresis at pH 3.5 of partial acid hydrolysates (2 h) of membranes (approximately 120 µg of protein) phosphorylated in the absence and presence of 2.2×10^{-7} M EGF. Spots 1 : labeled peptides; 2 : phosphotyrosine; 3 : phosphothreonine; 4 : phosphoserine; 5 : inorganic phosphate. The bars represent the origins.

tion) of the growth factor. Sinusoidal membranes have been shown to be derived from the hepatocyte blood face which accounts for nearly one-half of the total surface area of the cell (16). They thus can be expected to contain most of the EGF receptors.

We have also observed the stimulation by EGF of the phosphorylation of a 120 K component in the plasma membrane subfractions derived from the lateral and biliary faces of hepatocytes. Two reasons for this can be forwarded. The first is methodological : though minimal, some degree of cross-contamination of the subfractions may have occur (17, 18, 26). The second is physiological and gives regard to interdomain dynamics in a functional epithelium (26, 16). EGF receptors may be expected to diffuse freely from the blood sinusoidal region to be lateral one. Transepithelial transport processes from blood to bile may also result in the transfer of EGF receptors from the sinusoidal region to the canalicular one.

In agreement with the observations of Carpenter et al on A-431 cell membranes (6), both the basal and EGF-stimulated phosphorylation reactions required the presence of Mg^{2+} or Mn^{2+}. Mg^{2+} could substitute to 1 mM Mn^{2+} at a concentration of 5 mM. Phosphorylation assays were performed at 0°C, a condition which has been shown to sustain high level of phosphorylation by decreasing phosphatase activities (6). However, preliminary experiments show

that the EGF-stimulated phosphorylation of the 120 K protein also takes place at 33°C in sinusoidal membranes.

In contrast with the paucity of membrane phosphotyrosine in the basal state, EGF-stimulated phosphorylation of the 120 K protein is followed by a severalfold enrichment of liver membranes in this phosphoamino acid residue, as suggested by electrophoresis of partial acid hydrolysates of membrane subfractions. EGF may thus activate a membrane tyrosine protein kinase in normal mouse liver, a result in agreement with the pioneering work of Ushiro and Cohen on tumoral A-431 cell membranes (8).

The EGF-enhanced phosphorylation of mouse liver sinusoidal and lateral membranes also appears to take place on residues other than tyrosine in vitro. Comigration of phosphothreonine and phosphoserine markers with labeled acid-stable products after electrophoresis at pH 3.5 of partial acid hydrolysates of membranes is suggestive of the presence of threonine and serine as phosphate acceptors. Heat denaturation of eventual threonine and/or serine protein kinases contaminating the EGF preparation did not alter the pattern of phosphorylated components. Thus it may be expected that in addition to the activation of a membrane tyrosine protein kinase, EGF also controls, by direct or undirect ways, the activity of threonine and/or serine protein kinases in mouse liver membranes. Whether this observation is related to the in vitro conditions of the study, to the relatively low level of EGF receptors in liver (13, 14) as compared to A-431 cells where tyrosine is the main phosphate acceptor in vitro, or to particular physiological effects of EGF on liver, is as yet a matter for speculation. In addition, there is question upon the kinetics of formation of the phosphoamino acid residues which we observed in membrane hydrolysates since it is not implausible that the EGF-stimulated tyrosine protein kinase may in turn activate threonine and/or serine protein kinases in membranes.

The nature and function of the 120 K protein is not known. The EGF receptor in rat liver membranes has been solubilized after glutaraldehyde cross-linking of ^{125}I-labeled EGF and tentatively established to be an intrinsic glycoprotein of molecular weight 100 000 (15). A substantial improvement in the fraction of protein running in SDS gels was obtained after the samples were solubilized in a mixture containing 2 % SDS and 2 % Triton X-100, and incubated at room temperature for 40 min or more rather than at 100°C, before electrophoresis. This is indicative of hydrophobic properties (24). Whether the 120 K protein can be assimilated to the liver EGF receptor or corresponds to another substrate for membrane EGF-activated protein kinase(s) remains open to question.

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