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Solubilization of EGF Receptor with Triton X-100 Alters Stimulation of Tyrosine Residue Phosphorylation by EGF and Dimethyl Sulfoxide*

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In isolated hepatic membranes, epidermal growth factor (EGF) and the polar solvent dimethyl sulfoxide (Me_2SO) selectively stimulated the phosphorylation of the 170,000-dalton EGF receptor (p170) by 13.6 ± 2.0- and 10.9 ± 1.1-fold, respectively. The stimulation by maximally effective concentrations of the two substances was similar in rapidity of onset (less than 30 s at 0 °C), time course of phosphorylation, and tyrosine residue specificity. These maximal effects were not additive when the substances were combined, indicating that the same kinase/substrate combination is activated by each. The lectin concanavalin A, which inhibits EGF receptor binding, blocked the effect of EGF but not Me_2SO.

In membranes solubilized with Triton X-100, EGF stimulated p170 phosphorylation by 40- to 55-fold. Me_2SO also stimulated phosphorylation, indicating that it acts directly on the protein. However, the effect of the solvent was reduced by half. Additionally, Me_2SO blocks the effect of EGF in the solubilized preparation. A room temperature preincubation after addition of either substance was necessary for maximal stimulation of p170 phosphorylation in solubilized membranes. With EGF, 30-40 min was necessary; with Me_2SO, only 10 min was required. Thus, a secondary process appears to be involved in EGF receptor/kinase activation.

EGF[1] is a potent mitogen in hepatocytes (1) and a wide variety of other cultured cell types (2). When administered to animals, EGF stimulates DNA synthesis in liver (3) and other tissues (2). However, its role in the regulation of physiologic growth processes has not been established. In addition to its mitogenic properties, EGF causes a number of more rapid effects in cultured cells including alteration of cell structure (4) and ionic fluxes (5). A plasma membrane receptor, a 150,000- to 180,000-dalton glycoprotein (6-8), binds EGF with high affinity and is subsequently internalized (2).

Carpenter *et al.* have shown that EGF activates a protein kinase in membranes from a human epidermoid carcinoma line, A-431 (9, 10). Cohen *et al.* demonstrated that the EGF-dependent protein kinase activity and its major substrate site copurify with the EGF receptor by affinity chromatography, immunoprecipitation, and nondenaturing gel electrophoresis; the kinase and substrate appear to be intrinsic to the EGF receptor itself (11, 12). The phosphorylation takes place on tyrosine residues (13), suggesting that the kinase is related to the tyrosine residue protein kinase associated with oncogenic viruses (14-17), the normal cellular homologs of these enzymes (18), and the recently reported tyrosine residue kinases stimulated by platelet-derived growth factor (19) and insulin (20, 21). These associations suggest that this newly described class of protein kinases is linked to growth regulation.

We examined whether EGF receptor phosphorylation was altered in the well studied *in vivo* growth process, rat liver regeneration after partial hepatectomy. EGF stimulated phosphorylation of a 170,000-dalton protein (p170) (22), which we have identified as the hepatic EGF receptor (23). The EGF-stimulated phosphorylation of this protein was reduced in parallel with EGF binding during liver regeneration (22). This change resembled the reduction in EGF receptor number observed in cultured cells after treatment with EGF (2) or other growth factors (24) and suggested that endogenous growth factors produce a similar effect *in vivo* during liver regeneration.

During these studies we observed the stimulation of p170 tyrosine residue phosphorylation by the polar solvent Me_2SO, which was roughly similar in magnitude to that caused by EGF (25). This was interesting, because Me_2SO has a number of unexplained effects on cultured cells, including differentiation of murine erythroleukemia (Friend) cells (26). Me_2SO also produces more rapid effects, such as alteration of ion transport (27) and cellular structure (28). Changes in protein kinase activities could mediate some of these effects. Therefore, we characterized the EGF- and Me_2SO-induced stimulation of EGF receptor phosphorylation in liver membranes.

EXPERIMENTAL PROCEDURES

Materials—Male Sprague-Dawley rats (200-250 g) were obtained from Charles River Breeding Laboratories, Boston, MA and were fed and watered *ad libitum*. [γ-^{32}P]ATP (1000-3000 Ci/mmol) was purchased from New England Nuclear. Acrylamide, bisacrylamide, and phosphoamino acid standards were obtained from Sigma. Dimethyl sulfoxide was obtained from J. T. Baker Chemical Co. Precoated cellulose thin layer chromatography plates (0.1 mm) were purchased from E. Merck, Darmstadt, Germany. Initial experiments were performed using phosphotyrosine standard generously provided by Drs. J. Larner and C. Schwartz. Mouse EGF was purified by the method of Savage and Cohen (29).

Preparation of Membranes—Freshly prepared membranes were

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[1] The abbreviations used are: EGF, epidermal growth factor; Me_2SO, dimethyl sulfoxide; ConA, concanavalin A; SDS, sodium dodecyl sulfate; Pipes, 1,4-piperazinediethanesulfonic acid; src kinase, transforming protein kinase of Rous sarcoma virus.

used for all experiments. Rats were anesthetized with ether and the livers were excised between 8 and 9 a.m. Tissues were homogenized in 0.25 M sucrose/10 mM Tris-HCl, pH 8.0, with a Brinkmann Polytron apparatus. Homogenates were centrifuged at 30,000 × *g* for 7.5 min. The supernatant was centrifuged for 60 min at 105,000 × *g* to obtain the microsomal fraction, which was resuspended in 20 mM Pipes buffer, pH 7.0. Plasma membranes were made by the method of Touster *et al.* (30) by centrifuging microsomal fraction for 16 h in a discontinuous sucrose gradient. The plasma membranes were collected, centrifuged at 105,000 × *g*, and resuspended in 20 mM Pipes, pH 7.0. Protein was determined by the method of Lowry *et al.* (31).

Solubilization of Membranes—Microsomal fraction or plasma membranes were resuspended as usual at 10–20 mg of protein/ml and solubilized essentially as described by Cohen *et al.* (11). Triton X-100 and glycerol were added to final concentrations of 1 and 10%, respectively. The mixture was allowed to stand for 30 min at 0 °C and centrifuged for 60 min at 105,000 × *g* to remove debris.

Membrane Phosphorylation—Aliquots of membrane suspensions (50 μg of protein) were phosphorylated in a volume of 50 μl containing 50 mM Pipes/1 mM Na_2HPO_4 buffer, pH 7.0; 30 mM $MgCl_2$; EGF, Me_2SO or other test substances; and 1 μM [γ-^{32}P]ATP (5–10 μCi per tube). Triton X-100, when present, was kept to less than 0.3%. After addition of EGF or Me_2SO and preincubation of intact membranes for 10 min at 0 °C or solubilized membranes for 40 min at 21 °C, the reaction was started by addition of [γ-^{32}P]ATP and carried out for 60 s at 0 °C. The reaction was stopped with 25 μl of 9% SDS/0.05% bromphenol blue/15% glycerol/6% 2-mercaptoethanol/30 mM Tris-HCl, pH 7.8. Tubes were corked and heated to 100 °C for 3 min. Aliquots (50 μl) were subjected to electrophoresis in 8-mm wide lanes on SDS/8% polyacrylamide slab gels with a 3% stacking gel, essentially as described by Rudolph and Krueger (32). Gels were stained with Coomassie brilliant blue, destained, dried, and autoradiographed on Kodak XAR-5 film. Exposure time varied from 12 h to 3 days at −70 °C. Autoradiograms were scanned with a Kontes densitometer with peak integration performed on-line by a Hewlett-Packard reporting integrator.

Phosphoamino Acid Determination—Freshly prepared microsomal or plasma membranes (1.0 mg of protein) were phosphorylated in 300 μl under the conditions described above except that the ATP concentration was 15 μM (25 μCi per sample). The reaction (0 °C, 1 min) was stopped by addition of trichloroacetic acid to 50%. The precipitate was washed with 10% trichloroacetic acid (twice) and ether/ethanol (1:1). After drying, the samples were hydrolyzed in 6 N HCl in evacuated tubes for 3 h at 110 °C. The samples were lyophilized and resuspended in 25 μl of H_2O containing phosphoserine, phosphothreonine, and phosphotyrosine at 1 mg/ml. An aliquot of 5 μl was spotted on cellulose thin layer plates, and the amino acids were separated by the two-dimensional procedure of Hunter and Sefton (15). After electrophoresis (900 V, 45 min) in glacial acetic/88% formic acid/H_2O, 78:25:897, v/v, pH 1.9, ascending chromatography was performed using isobutyric acid/0.5 M NH_4OH, 5:3, v/v. The plates were dried and subjected to autoradiography as described above. Radioactive spots were scraped from the plates, and the ^{32}P-labeled amino acids were eluted in 1 N NaOH, neutralized, and counted.

Analysis of p170 was studied by phosphorylating membranes (200–600 μg of protein) with 1 μM [γ-^{32}P]ATP (80 μCi) in 200–300 μl and subjecting it to SDS/polyacrylamide gel electrophoresis, using 4–8 gel lanes. The gel was rapidly stained and destained, and the p170 band was identified and cut out. The slice was placed in a tube gel apparatus and eluted electrophoretically for 20 h into a dialysis bag. One mg of bovine serum albumin was added and the protein was precipitated with 15% trichloroacetic acid. Subsequent steps were identical with those described above. The remainder of the gel was subsequently dried and subjected to autoradiography to assess accuracy of phosphoprotein excision. Extending the electrophoretic elution to 40 or 64 h did not increase the yield. Analysis of replicate samples yielded reproducible results even when low counts per min were present; the coefficient of variation averaged 10%.

ATP Analysis—The phosphorylation reaction was carried out exactly as described above, but the stop solution was replaced with 500 μl of 0.5 N $HClO_4$. The samples were kept on ice for 10 min and centrifuged to remove precipitated protein. The supernatants were combined with equal volumes of 0.5 N KOH. The precipitates were removed by centrifugation. Unlabeled ATP carrier was added to the supernatants, which were subjected to thin layer chromatography on cellulose polyethyleneimine plates (Sybron/Brinkman) in 0.5 M $LiCl_2$/2.0 N acetic acid. The plates were dried and the ATP spots were identified under UV light, and the rest of the lanes were divided into several segments. Each region was transferred to a scintillation vial; Scintiverse 1 (Fisher) was added and the samples were analyzed by liquid scintillation counting. The proportion of ATP remaining was the counts per min in the ATP spot divided by the total counts per min in all the segments.

RESULTS

p170 Phosphorylation in Intact Membranes—Previous work had indicated that the effects of EGF and Me_2SO on p170 phosphorylation were roughly equivalent. We further compared the magnitude of maximal stimulation, the time course of phosphorylation, and the rapidity of onset of action of the two substances. Liver microsomal fraction or plasma membrane preparations were phosphorylated (1 min, 0 °C) in presence or absence of EGF (1 μg/ml) or Me_2SO (20% v/v), maximally effective concentrations. Phosphorylation of p170 was stimulated 13.6 ± 2.0-fold and 10.9 ± 1.1-fold, respectively (means ± S.E., n = 12). Two other solvents were tested for comparison. Acetone (20%) was approximately as effective as Me_2SO, and ethanol (10%) caused partial stimulation (data not shown).

The time courses of p170 phosphorylation were compared in the presence or absence of EGF (1 μg/ml) or Me_2SO (20%). Both substances stimulated p170 phosphorylation by 15 s, and the degree of activation by each was similar throughout (Table I). The time required for the onset of action was compared by adding EGF or Me_2SO to membranes and preincubating at 0 °C for periods varying from 15 s to 10 min. Phosphorylation (1 min, 0 °C) was then started by addition of [γ-^{32}P]ATP. Each substance caused nearly maximal activation at 15 s and maximal activation at 30 s and all longer preincubations (data not shown). The onset of action of each compound is clearly very rapid.

p170 Phosphorylation in Solubilized Membranes—Microsomal fraction was solubilized in 1% Triton X-100/10% glycerol. After centrifugation, 67% of the protein remained in the supernatant (solubilized fraction). This was assayed for phosphorylating activity and compared to the intact membranes at identical protein concentrations. Solubilized membranes required preincubation for 40 min at 21 °C to observe maximum stimulation of kinase activity (see below); this treatment was without effect on intact membranes, which were, therefore, preincubated in the ususal manner (10 min, 0 °C). Both EGF (1 μg/ml) and Me_2SO (20% v/v) stimulated p170 phosphorylation in intact and solubilized membranes (Fig. 1). Densitometry revealed that the substances had similar effects in intact membranes as previously observed. In solubilized membranes, basal p170 phosphorylation was reduced; EGF and Me_2SO stimulated p170 phosphorylation by 40-fold and 9-fold, respectively. EGF-stimulated p170 phosphorylation was 2.7-fold greater than in intact membranes; Me_2SO-stimulated phosphorylation was reduced by half. Additionally, Me_2SO stimulated phosphorylation of two protein bands other than p170 in the solubilized preparation.

The magnitude of these effects was verified by scintillation counting. The p170 regions were cut out of the dried gel,

TABLE I

Effect of EGF or Me_2SO on the time course of p170 phosphorylation in hepatic plasma membranes

Hepatic plasma membranes were phosphorylated with 10 μM [γ-^{32}P]ATP for 15, 30, or 60 s in the presence of EGF or Me_2SO. p170 phosphorylation was measured by densitometry of autoradiograms.

Addition	15 s	30 s	60 s
		arbitrary units	
None	26	44	74
EGF (1 μg/ml)	228	320	415
Me_2SO (20%)	229	306	439

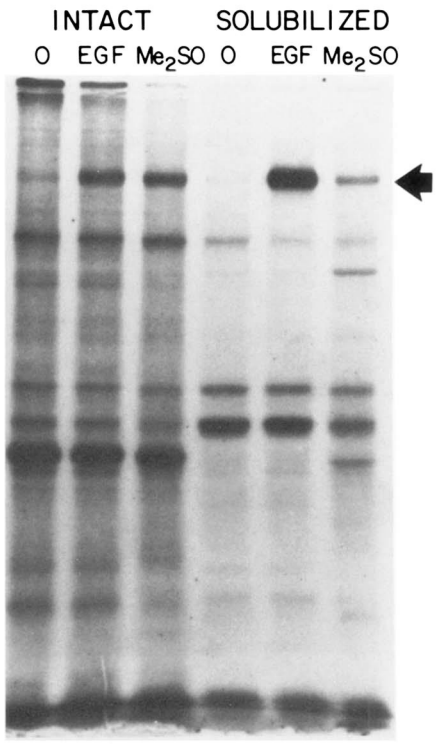

FIG. 1. **Effect of EGF or Me_2SO on protein phosphorylation in liver microsomal fraction.** Membranes (50 μg of protein) were preincubated (10 min at 0 °C for intact membranes or 40 min at 21 °C for solubilized preparation) with EGF (1 μg/ml) or Me_2SO (20% v/v) and phosphorylated with [γ-^{32}P]ATP (60 s, 0 °C). Samples were subjected to electrophoresis on an SDS/polyacrylamide gel (8% polyacrylamide) and an autoradiogram was prepared. *Arrow*, p170.

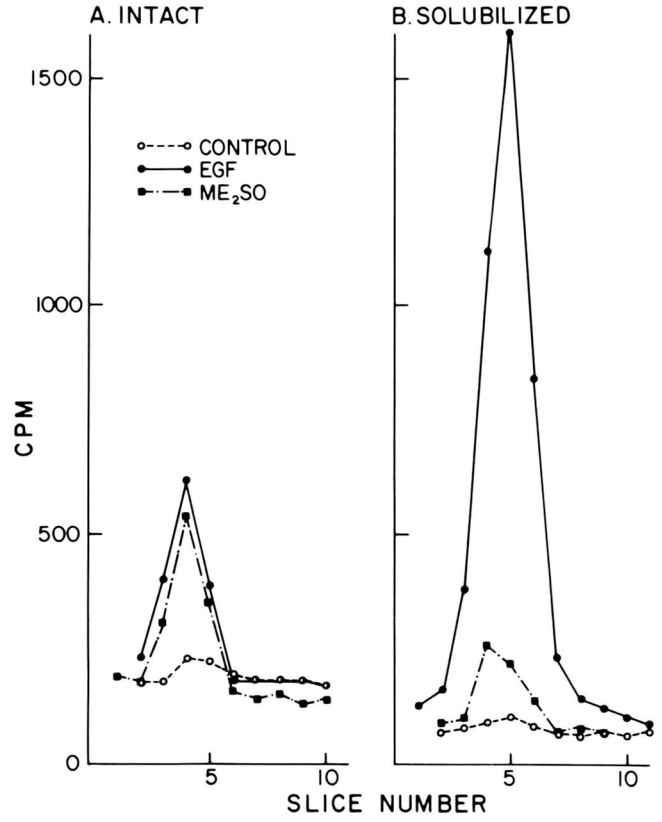

FIG. 2. **Effect of EGF or Me_2SO on p170 phosphorylation: analysis by liquid scintillation counting.** The p170 region of the dried gel (see Fig. 1) was reconstituted and sliced into serial 1-mm blocks, which were digested with 0.5 ml of 9:1 NCS/H_2O (v/v) for 60 min at 60 °C. 5 ml of toluene/Permafluor (100:4, v/v) were added for liquid scintillation counting.

reconstituted in destaining solution, sliced into serial 1-mm blocks, digested, and analyzed by liquid scintillation counting. The results (Fig. 2) were similar to those obtained by densitometry.

Similar results were obtained with density gradient purified liver plasma membranes, a preparation enriched in EGF receptors. EGF and Me_2SO stimulated p170 phosphorylation to a similar degree in intact membranes; Triton solubilization doubled the effect of EGF and reduced the effect of Me_2SO (data not shown). In A-431 cell membranes, Me_2SO stimulated p170 phosphorylation but was less effective than EGF; solubilization enhanced the effect of EGF.[2]

To assess the possibility that altered rates of ATP hydrolysis affected phosphorylation, two types of experiments were performed. First, intact and solubilized membranes were phosphorylated in the presence or absence of EGF or Me_2SO. The reaction was terminated with perchloric acid, and [γ-^{32}P]ATP was analyzed by thin layer chromatography as described under "Experimental Procedures." The proportion of the total radioactivity found in the ATP spot was calculated. ATP hydrolysis was unaffected by solubilization, EGF, or Me_2SO. The mean ATP value ($N = 3$ experiments) ranged from 74 to 80% of the total counts per min. (data not shown). Second, parallel experiments were performed with 25 μM ATP as well as the usual 1 μM. Similar effects on p170 phosphorylation were observed in each case (data not shown). These experiments demonstrated that differences in ATP hydrolysis did not contribute to the observed effects.

As mentioned above, stimulation of solubilized p170 phosphorylation required preincubation at 21 °C; this was not necessary in intact membranes. To determine the optimal conditions for phosphorylation, solubilized microsomal fraction was preincubated with 1 μg/ml of EGF or 20% Me_2SO at 21 °C for varying periods of time, chilled to 0 °C and phosphorylated (1 min, 0 °C). Preincubation with EGF or Me_2SO enhanced p170 phosphorylation in a time-dependent manner. The effect of EGF reached a maximum of 55-fold after 30 min (Fig. 3). Separate experiments showed that activation by EGF was complete after 30–40 min (data not shown). The effect of Me_2SO averaged 10- to 11-fold and was maximal at 10 min (Fig. 3). Preincubation with EGF for 40 min at 0 °C did not stimulate p170 phosphorylation; preincubation without EGF at 21 °C for 40 min, chilling the sample to 0 °C, and adding EGF prior to phosphorylation was also without effect (data not shown).

Combined Effects of EGF and Me_2SO—In intact membranes, the combination of 20% Me_2SO plus 1 μg/ml of EGF resulted in no more p170 phosphorylation than either substance alone (Table II). This indicated that the same kinase and substrate are affected by each. In solubilized membranes, the combination had a different effect; 20% Me_2SO increased p170 phosphorylation but nearly abolished the enhanced stimulation by EGF (Table II). This may result from the reduction of EGF binding affinity caused by Me_2SO.[2]

Effect of Concavavalin A—The possible differences between the action of EGF and Me_2SO were further explored by pretreating liver membranes with ConA. This lectin inhibits the binding of EGF to membranes (8) and was expected to affect EGF-dependent phosphorylation. Microsomal fraction was incubated (21 °C, 30 min) with or without ConA (170 μg/ml), chilled, further incubated (10 min, 0 °C) with EGF (1 μg/

[2] R. A. Rubin and H. S. Earp, unpublished results.

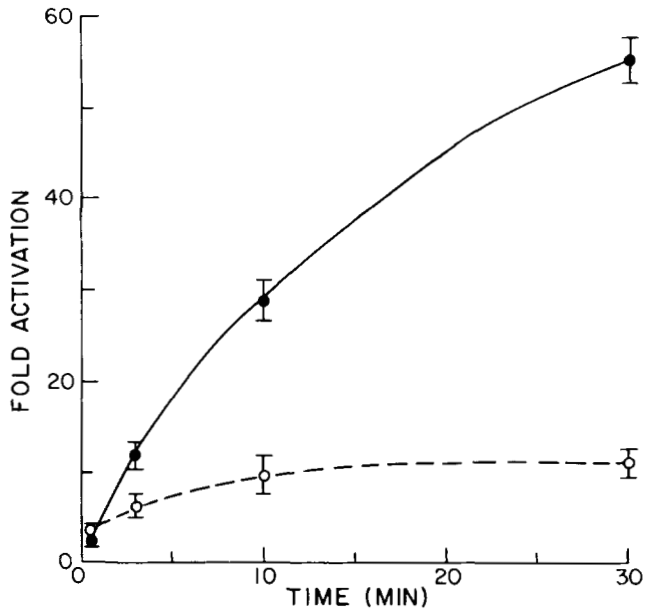

FIG. 3. **Effect of preincubation on EGF- and Me_2SO-stimulated p170 phosphorylation in solubilized membranes.** Solubilized microsomal fraction was preincubated with or without EGF (1 μg/ml) or Me_2SO (20%) for various periods at 21 °C. The samples were then chilled and phosphorylated for 1 min at 0 °C. Gels and autoradiograms were prepared and analyzed as described under "Experimental Procedures." Each experimental value was expressed as "fold elevation," the ratio to the control value. (Control values, determined separately for each preparation, were the average from four aliquots with no EGF or Me_2SO.) The results shown are the means ± S.E. from four membrane preparations assayed in parallel. ●, EGF; ○, Me_2SO.

ml) or Me_2SO (20% v/v), and phosphorylated (1 min, 0 °C). ConA did not measurably affect basal p170 phosphorylation but almost completely abolished the stimulation caused by EGF (Table III). When EGF was incubated with the membranes first for 1 min at 0 °C, subsequent incubation with ConA did not affect phosphorylation (data not shown). Interestingly, the lectin did not reduce the phosphorylation caused by Me_2SO (Table III).

Phosphoamino Acid Specificity—We determined the time courses of total serine and tyrosine phosphorylation in microsomal fraction in the presence or absence of EGF (1 μg/ml). Membrane proteins were phosphorylated, precipitated, hydrolyzed, and analyzed by two-dimensional electrophoresis and chromatography followed by autoradiography and scintillation counting as described under "Experimental Procedures." Tyrosine residue phosphorylation was maximal by 1 min and was clearly enhanced by EGF; phosphorylation of serine continued to increase for 3–4 min and was not altered by EGF (Fig. 4).

Intact microsomal fraction was phosphorylated for 1 min and total membrane protein was analyzed for ^{32}P-phosphoamino acid content. Serine was the predominant phosphorylated amino acid residue; threonine and tyrosine were also phosphorylated in unstimulated membranes (Table IV). Both EGF (1 μg/ml) and Me_2SO (20%) doubled tyrosine residue labeling in these membranes (Table IV). EGF did not alter serine or threonine phosphorylation; in contrast, Me_2SO caused a 46% fall in serine and a 25% fall in threonine phosphorylation, resulting in reduced total incorporation. This reduction was a rather general effect; autoradiograms showed that Me_2SO moderately reduced phosphorylation of many proteins (*e.g.* Fig. 1).

We also determined the effects of EGF and Me_2SO on the phosphoamino acid content of p170. Intact and solubilized plasma membranes were phosphorylated and p170 was electrophoretically isolated and analyzed as described under "Experimental Procedures." Autoradiograms from one exper-

TABLE II

Phosphorylation of p170 in the presence of both EGF and Me_2SO

Intact or solubilized microsomal fraction was phosphorylated for 1 min at 0 °C with various concentrations of EGF in the presence or absence of 20% Me_2SO, which was added first. Preincubation was for 10 min at 0 °C for intact membranes and for 40 min at 21 °C for the solubilized preparation. Gels and autoradiograms were prepared and analyzed as described under "Experimental Procedures."

Membrane preparation	Me_2SO (20%)	EGF (ng/ml)			
		0	10	100	1000
		arbitrary units			
Intact	−	127	177	614	624
	+	706	496	609	664
Solubilized	−	133	145	2567	2942
	+	362	230	382	523

TABLE III

Effect of concanavalin A on p170 phosphorylation

Microsomal fraction was preincubated (30 min, 21 °C) with or without concanavalin A (170 μg/ml), chilled to 0 °C, incubated for 10 min at 0 °C with EGF (1 μg/ml) or Me_2SO (20%), and phosphorylated for 60 s. p170 phosphorylation was determined as described under "Experimental Procedures."

Pretreatment	Condition		
	H_2O	EGF (1 μg/ml)	Me_2SO (20%)
	arbitrary units		
H_2O	31	802	308
Concanavalin A	42	105	276

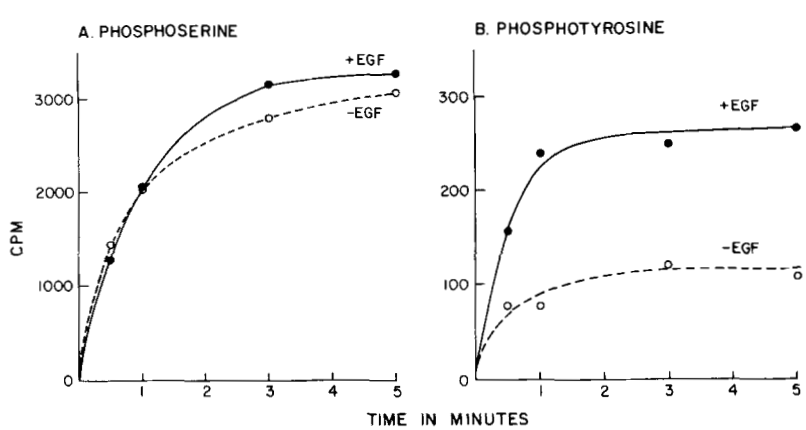

FIG. 4. **Time courses of serine and tyrosine residue phosphorylation.** Microsomal fraction (1.0 mg of protein) was phosphorylated in the presence of 15 μM [γ-^{32}P]ATP (25 μCi per sample) for various periods. Phosphoamino acid analysis was performed as described under "Experimental Procedures."

iment are shown in Fig. 5, and cumulative data from scintillation counting are shown in Table V. In untreated intact membranes, p170 was phosphorylated on serine, threonine, and tyrosine; serine was the most common site. EGF selectively stimulated tyrosine phosphorylation of p170 by 6- to 8-fold. Me_2SO increased phosphotyrosine by a similar amount. In solubilized membranes, basal phosphotyrosine was higher than in intact membranes and was the predominant phosphoamino acid. EGF stimulated tyrosine phosphorylation of solubilized p170 by 30-fold. This corresponds to the large activation of total p170 phosphorylation observed in solubilized membranes. Me_2SO also stimulated tyrosine phosphorylation of solubilized p170 but not as much as did EGF. Thus, the effects of these substances on the phosphotyrosine content of p170 parallelled their effects on total p170 phosphorylation (*cf.* Figs. 1 and 2).

TABLE IV

Effect of EGF and Me_2SO on phosphoamino acid content of hepatic membranes

Intact membrane preparations were phosphorylated in the presence of 5 μM [γ-^{32}P]ATP. Phosphoamino acid analysis was performed as described under "Experimental Procedures." Relative abundance (expressed as a per cent) is the counts per min incorporated in the individual spot divided by the total counts per min in all three spots from the sample (the counts per min data are not shown). The change produced by EGF or Me_2SO is also expressed as the counts per min in the individual spot from a sample phosphorylated with EGF (or Me_2SO) divided by the control value. The means ± S.E. of the individual samples are shown (EGF, $N = 17$; Me_2SO, $N = 6$). Statistical significance was determined by paired t test of the counts per min data and is indicated near the appropriate ratio value.

	^{32}P-Phosphoamino Acid		
	P-Ser	P-Thr	P-Tyr
A. Effect of EGF			
% Abundance			
−EGF	78.9 ± 1.5%	16.8 ± 1.3%	4.3 ± 0.3%
+EGF	76.6 ± 1.7%	16.2 ± 1.4%	7.2 ± 0.8%
Cpm ratio: +EGF/−EGF	1.10 ± 0.06	1.09 ± 0.09	2.00 ± 0.27[a]
B. Effect of Me_2SO			
% Abundance			
−Me_2SO	79.5 ± 1.1%	18.9 ± 0.9%	1.62 ± 0.45%
+Me_2SO	68.8 ± 2.2%	22.6 ± 1.4%	8.65 ± 3.10%
Cpm ratio: +Me_2SO/−Me_2SO	0.54 ± 0.02[b]	0.75 ± 0.14	3.28 ± 0.43[b]

[a] $p < 0.01$.
[b] $p < 0.02$.

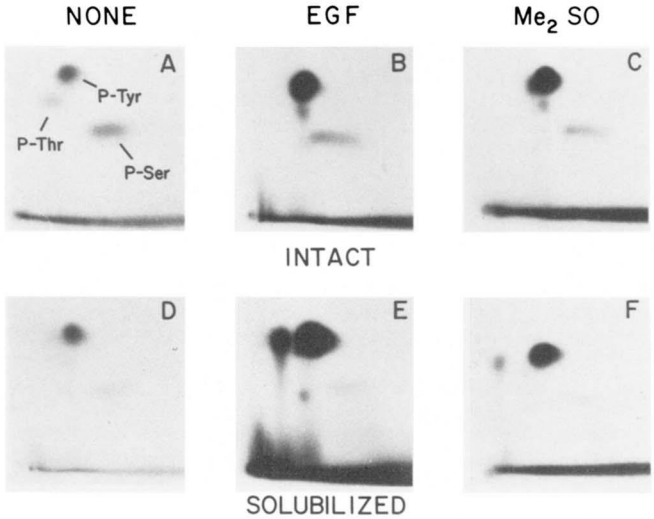

FIG. 5. **Effect of EGF or Me_2SO on phosphoamino acid composition of p170.** Intact (*A–C*) or solubilized (*D–F*) plasma membrane was preincubated with or without EGF (1 μg/ml) or Me_2SO (20%) and phosphorylated (1 min, 0 °C). p170 was isolated on SDS/polyacrylamide gels, cut out, eluted electrophoretically, and hydrolyzed. Phosphoamino acids were analyzed by two-dimensional electrophoresis and chromatography and detected by autoradiography.

TABLE V

Phosphoamino acid content of electrophoretically purified p170

Microsomal fraction or plasma membrane was phosphorylated, and p170 was isolated and analyzed as described under "Experimental Procedures." The phosphoamino acid abundance and ratio of stimulation by EGF were calculated as described in Table II.

Membrane preparation	P-Ser	P-Thr	P-Tyr
Microsomal fraction ($N = 4$)			
% Abundance			
−EGF	69.2 ± 4.4%	13.5 ± 3.7%	17.3 ± 5.9%
+EGF	39.3 ± 3.6%	6.5 ± 2.1%	54.3 ± 5.0%
Ratio: +EGF/−EGF	1.2 ± 0.3	0.95 ± 0.19	8.28 ± 3.01
Intact plasma membranes ($N = 2$)			
% Abundance			
−EGF	45%	23%	32%
+EGF	22%	12%	66%
Ratio: +EGF/−EGF	1.10	1.05	5.95
Solubilized plasma membranes ($N = 2$)			
% Abundance			
−EGF	26%	8%	66%
+EGF	2%	ND[a]	98%
Ratio: +EGF/−EGF	0.9	ND	30.1

[a] ND, not detectable.

It is of interest that solubilization reduced basal p170 serine phosphorylation by 75% (Table V). Total membrane phosphoserine is reduced as well (data not shown), and this is reflected by reduced incorporation into most phosphoprotein bands (similar to Fig. 1). This general effect indicates that serine kinases are inhibited by solubilization or remain in the pellet. In addition, neither EGF nor Me_2SO increased p170 serine phosphorylation. These findings indicate that p170 serine phosphorylation is mediated by a kinase other than the EGF-stimulated tyrosine residue kinase.

DISCUSSION

Tyrosine residue phosphorylation of a 170,000-dalton hepatic plasma membrane protein (p170) is stimulated by EGF and Me_2SO. p170 comigrates with the EGF receptor identified by chemical cross-linking of ^{125}I-EGF to liver membranes (23) and with the EGF receptor/kinase complex of A-431 cell membranes.[2] Phosphorylation stimulated by EGF and Me_2SO in intact hepatic membranes is similar in selectivity, magnitude, rapidity of onset, time course, and tyrosine residue specificity. The combination of maximally effective concentrations of EGF and Me_2SO did not further stimulate phosphorylation. These findings indicate that EGF and Me_2SO are stimulating the same kinase/substrate combination.

Solubilization of the membrane with Triton X-100 altered the effects of EGF and Me_2SO. Stimulation of p170 phosphorylation by EGF was enhanced, while the effect of Me_2SO was reduced. The increased effect of EGF could result from releasing the receptor/kinase from some inhibitor or constraint imposed by the cell membrane. Interestingly, Triton solubilization also enhances the stimulation by insulin and platelet-derived growth factor of tyrosine phosphorylation of their respective receptors (33, 34). The persistence of Me_2SO-stimulated p170 phosphorylation in this preparation indicates that the solvent affects the receptor directly; effects of Me_2SO on the membrane, such as reduction of fluidity (35), cannot account for this activation. The membrane or its constituent lipids may, however, play a partial role, since the solvent was

less effective in the solubilized preparation. Me_2SO strongly inhibits the effect of EGF in solubilized membranes. This may result from alterations in EGF binding; Me_2SO reduces the affinity of the hepatic EGF receptor for ^{125}I-EGF,[2] and the affinity of the EGF receptor is reduced by solubilization as well (36). The combination of 1% Triton X-100 and 20% Me_2SO may reduce EGF binding to the point where it no longer stimulates phosphorylation.

The solubilized enzyme requires preincubation for 30–40 min at 21 °C with EGF for maximum activation. Increasing the EGF concentration to 10 μg/ml did not accelerate this process,[2] indicating that slow binding was not solely responsible for the delay. Stimulation with Me_2SO also requires preincubation for 10 min at 21 °C, suggesting that a similar secondary process is needed. In intact membranes, this step appears to be very rapid and is not temperature-dependent. When the normal milieu of the receptor is disrupted by solubilization, the secondary process is retarded. Association of receptor monomers or other disaggregated components, an enzymatic step, or a slow conformational change could be occurring during this period. EGF-induced stimulation of the solubilized A-431 receptor also required a room temperature preincubation, but full activation was observed after only 4 min (11). Perhaps the higher concentration of receptors in the A-431 membrane allows more rapid aggregation or other interaction among soluble EGF-receptor complexes. EGF stimulates phosphorylation of highly purified receptor (11), suggesting that the receptor/kinase itself is the only protein component involved in this step.

In liver membranes, the EGF receptor was phosphorylated on serine, threonine, and tyrosine residues. In intact A-431 cells labeled with $^{32}P_i$, the EGF receptor also contained all three phosphoamino acids (37). Thus, phosphorylation of the liver EGF receptor in membranes presumably reflects events that occur in living cells. Serine phosphorylation of the src kinase, another autophosphorylating tyrosine kinase, has also been reported (38). Solubilization of the membrane with Triton X-100 stimulates basal tyrosine phosphorylation and enhances EGF-dependent tyrosine phosphorylation. Serine and threonine phosphorylation are reduced, indicating that separate kinases are responsible for EGF receptor phosphorylation at different sites.

The high concentration of Me_2SO required for maximum stimulation (15–25%) and the similar effects of acetone and ethanol makes it unlikely that a specific site for Me_2SO is involved in the activation of p170 phosphorylation. Phosphorylase kinase, a serine residue kinase, is also activated by high concentrations of Me_2SO, acetone, or ethanol. Presumably, solvents can activate some protein kinases by producing a conformational change.

A biological response may be elicited at lower concentrations of an active agent than are needed to cause a measurable biochemical response. Higher concentrations of EGF are needed for maximal kinase activation in membranes than are required for full proliferative response in culture. This is consistent with the presence of "spare receptors" for EGF, which is maximally mitogenic at only 20% receptor occupancy (39). Thus, it is possible that submaximal stimulation of tyrosine phosphorylation may mediate EGF action. While it is unknown whether tyrosine phosphorylation is stimulated by the low concentrations of Me_2SO (1–2%) used to differentiate murine erythroleukemia cells, further studies in cell culture seem warranted.

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