An Electrophoretic Study of Endogenous Phosphorylation in vitro of the Polypeptides of Microsomal Membrane Fractions of Mouse Liver

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1. The patterns of phosphopolypeptides produced by endogenous phosphorylation in vitro of rough- and smooth-membrane fractions of the microsomal fraction of mouse liver were studied by radioautographic analysis of sodium dodecyl sulphate/polyacrylamide-gel electrophoretograms. 2. A minimum of 17 polypeptides of both rough- and smooth-microsomal-membrane fractions were phosphorylated by using [γ-32P]-ATP as the phosphate donor; only minor differences in phosphorylation pattern between the two membrane fractions were detected. 3. Phosphorylation in vitro by [γ-32P]ATP was markedly stimulated by Mg²⁺, but not by cyclic AMP, cyclic GMP or Ca²⁺. The phosphorylation of certain polypeptides was preferentially stimulated by Mg²⁺. Addition of cyclic AMP resulted in a decrease in the amount of ³²P detected in one polypeptide of mol.wt. approx. 56000, present in both the rough- and smooth-membrane fractions. 4. [γ-32P]GTP was found to be a relatively poor donor of ³²P as compared with [γ-3²P]ATP. However, incubation of rough- and smooth-membrane fractions with this compound resulted in the phosphorylation of one polypeptide of mol.wt. approx. 96000 that was scarcely or not at all phosphorylated by [γ-3²P]ATP. 5. Under the conditions of incubation used, appreciable incorporation of ³²P from [γ-3²P]ATP occurred into products migrating at the front of the electrophoretograms; these products were identified as being principally comprised of 1-phosphatidylinositol-4-phosphate. Incorporation of ³²P into this lipid was also markedly stimulated by Mg²⁺. 6. The overall results show that a considerable number of polypeptides of the rough- and smooth-microsomal-membrane fractions of mouse liver may be phosphorylated in vitro and indicate that the enzymes responsible are principally non-cyclic AMP-dependent protein kinases.

A number of investigations have demonstrated phosphorylation of polypeptides of various membranes by endogenous protein kinases (reviewed by Rubin & Rosen, 1975; Hosey & Tao, 1977; Lam & Kasper, 1979). (The term 'polypeptide' is used here to describe components resolved by SDS/polyacrylamide-gel electrophoresis, as very often it cannot be excluded that this type of electrophoresis may have dissociated native membrane proteins into polypeptide subunits.) Several studies have shown that a number of polypeptides of microsomal membrane fractions of rat liver may be phosphorylated in vitro by endogenous protein kinases (Jergil & Ohlsson, 1974; Sharma et al., 1976; Sharma et al., 1978; Lam & Kasper, 1979); phosphorylation of certain polypeptides of the microsomal fraction of bovine corpus luteum has also been studied (Hardie & Stansfield, 1977). The work of Sommarin & Jergil (1978) has suggested that several protein kinases may be involved in the endogenous phosphorylation of polypeptides of the microsomal membranes of rat liver. In our study of the phosphorylation of microsomal membrane fractions of rat liver (Sharma et al., 1978), we also reported that the polypeptides of the microsomal membranes of mouse liver showed a pattern of phosphorylation similar to that exhibited by the corresponding fractions of rat liver. A feature of that study was the relatively large number of polypeptides observed to be phosphorylated both in vitro and in vivo; whereas other studies have revealed anywhere from two to five phosphorylated polypeptides in the microsomal-membrane fractions of rat liver (Jergil & Ohlsson, 1974; Sharma et al., 1976; Lam & Kasper, 1979), our work indicated

Abbreviation used: SDS, sodium dodecyl sulphate.  
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that a minimum of 20 polypeptides was phosphorylated. In the present paper we report the results of analyses of the effects of various factors (phosphate donors, cyclic nucleotides, metal cofactors etc.) on the patterns of phosphorylation in vitro of the polypeptides of rough- and smooth-microsomal-membrane fractions of mouse liver. The results indicate that the enzymes responsible for endogenous phosphorylation of polypeptides of microsomal-membrane fractions of mouse liver are primarily non-cyclic-nucleotide-dependent protein kinases. The results also re-emphasize the complexity (in terms of number) of the polypeptides of microsomal membranes that may be phosphorylated in vitro. In addition, we show that appreciable incorporation of $^{32}$P into lipid products migrating at the front of SDS/polyacrylamide-gel electrophotograms and identified as principally 1-phosphatidylinositol 4-phosphate also occurs during the conditions used for phosphorylation of microsomal membranes in vitro. A preliminary account of certain aspects of this work has been presented (Behar-Bannelier & Murray, 1979).

Experimental

Animals

Female Swiss mice of approx. 20g body weight were obtained from Connaught Medical Research Laboratories, Toronto, Ont., Canada. The animals were maintained on Purina chow and water ad libitum, until 16 h before use, at which time the chow was withdrawn.

Preparation of microsomal-membrane fractions

The mice were killed by decapitation. The livers were immediately removed and washed in ice-cold 0.20 M-STKM buffer [0.20 M-sucrose containing 0.05 M-Tris/HCl, pH 7.5, 0.025 M-KCl and 0.005 M-MgCl$_2$]. Total microsomal and rough- and smooth-membrane fractions were prepared as described previously (Sharma et al., 1978; Behar-Bannelier et al., 1979); removal of ribosomes from these fractions was also performed as detailed by Sharma et al. (1978). For all of the conditions of phosphorylation studied, a preliminary experiment was performed on portions of the fresh unfrozen total microsomal fraction. The remainsders of the total microsomal fraction and the rough- and smooth-membrane fractions were stored at -70°C. All fractions were thawed only once, just before use, which was within several days of their preparation. The results described here are based on analyses of three to four preparations of such membrane fractions per condition of phosphorylation studied, each preparation being derived from the livers of individual mice or pairs of mice.

Phosphorylation in vitro

The standard assay contained microsomal-membrane protein (up to 250 $\mu$g), 50 mM-Tris/HCl, pH 7.4, 10 mM-NaF, 5 mM-MgCl$_2$ and 22 $\mu$M-[y-$^{32}$P]-ATP (New England Nuclear Corp., Boston, MA, U.S.A.) (approx. 10000 Ci/mol) in a final volume of 100$\mu$L. Incubations were performed at 30°C for 2 min. The reactions were stopped by the addition of 100$\mu$L of ice-cold 10% (w/v) trichloroacetic acid. The precipitated protein was washed twice with 200$\mu$L of ice-cold trichloroacetic acid and then twice with 500$\mu$L of ice-cold 0.20 M-STKM buffer. In various experiments the concentrations of Mg$^{2+}$ and ATP in the above reaction mixture were varied systematically; the effects of addition of cyclic AMP and cyclic GMP and of replacing Mg$^{2+}$ with Ca$^{2+}$ were also investigated. The ranges of concentrations of the above compounds used are detailed in the Results section. In certain experiments [$\gamma$-$^{32}$P]GTP (New England Nuclear) was also used as a phosphate donor; its specific radioactivity was adjusted to that of the radioactive ATP used in the standard assay.

Protein determination

The protein contents of the various microsomal fractions used were measured as described by Lowry et al. (1951), with bovine serum albumin as standard.

Determination of radioactivity

This was performed as described previously (Sharma et al., 1978).

SDS/polyacrylamide-gel electrophoresis, radioautography and counting of radioactivity in gels

The first two of these procedures were also performed as described previously (Sharma et al., 1978), by using 12.5% (w/v) polyacrylamide gels. The various molecular-weight-standard proteins used were as described by Sharma et al. (1979). In certain experiments the radioactivity in selected areas or bands [i.e. the radioactive zone at the front of the electrophoretograms and also the polypeptide of approx. 12000 mol. wt. whose phosphorylation was markedly increased by addition of Mg$^{2+}$ (see the Results section)] of stained gels was measured. This was done by cutting out the appropriate areas of gels, slicing them into 4 mm-thick segments and digesting these in scintillation vials containing 0.5 ml of Protosol (New England Nuclear)/toluene/water (9:10:1, by vol.) at 60°C for 20 h. A portion (10 ml) of a Liquifluor (New England Nuclear)/toluene (1:24, v/v) mixture was then added and the vials placed in an incubator at 37°C for a further 20 h before counting for radioactivity. The radioactivity present in the other phosphopolypeptide bands was not measured because their separation
Analyses by electrophoresis of moieties containing $^{32}$P

Certain samples of $^{32}$P-labelled membrane fractions were extracted for lipid (see below). Portions of the dried lipid extracts, of the non-lipid residues and of the original non-extracted membrane fractions were added separately to 0.3 ml of 2 M HCl and subjected to partial hydrolysis in sealed tubes at 110°C for 5 h (cf. Jergil & Ohlsson, 1974; Sharma et al., 1978). The hydrolysates were dried under vacuum, resuspended and added separately to 2000 g super.

Extraction of lipids

This was performed by the method of Dawson & Eichberg (1965). Labelled membrane fractions were extracted three times at room temperature with 10 vol. of chloroform/methanol (2:1, v/v). In each case, the insoluble material was sedimented by centrifugation at 2000 $g_{max}$ for 10 min and the supernatant decanted. The supernatants were pooled and denoted 'extract I'. The residues were further extracted three times at room temperature for 20 min with 4 vol. of chloroform/methanol (2:1, v/v) containing 0.25% (v/v) conc. HCl. These extracts were washed once with 0.5 ml of chloroform/methanol/1 M HCl (3:48:47, by vol.). The acidified chloroform phase was filtered and was neutralized by brief exposure to gaseous NH$_3$. This extract was denoted 'extract II'.

T.l.c. of lipids

Extracts I and II were dried under N$_2$ and resuspended in chloroform/methanol/water (75:25:2, by vol.). Small portions of both extracts were taken for determination of radioactivity. As this revealed that most of the radioactivity was present in extract II (see the Results section), only this fraction was subjected to analysis by t.l.c., by the procedure of Gonzalez-Sastre & Folch-Pi (1968). Glass plates were coated with silica-gel HR (Merck, Darmstadt, Germany) (0.25 mm thickness) suspended in a 1% solution of potassium oxalate. The plates were developed in chloroform/methanol/aq. NH$_3$ (9:7:2, by vol.) and subsequently air-dried and then subjected to radioautography. After development of the radioautograms (1–2 weeks) the lipids were revealed by spraying with the reagent of Vaskovsky & Kostetsky (1968). Standards of 1-(3-sn-phosphatidyl)inositol, 1-phosphatidylinositol 4-phosphate and 1-phosphatidylinositol 3,4-bisphosphate were generously supplied by Dr. F. B. Palmer, Dalhousie University, Halifax, Nova Scotia, Canada.

Results

Tracks 3 and 8 respectively of Fig. 1 show the patterns of phosphorylation of the smooth- and rough-microsomal-membrane fractions produced under the standard incubation conditions (5 mM Mg$^{2+}$) used in these experiments. A minimum of 17 radioactive zones is visible in the smooth-membrane fraction, and approximately the same number of zones is seen in the rough-membrane fraction. Phosphorylation of zones corresponding to approx. 62 000 and 35 000 mol wt. (indicated by the white dots in track 8) was consistently greater in the rough-membrane fraction (three experiments), whereas the phosphorylation of the zone of approx. 41 000 mol wt. (indicated by the white dot in track 3) was consistently greater in the smooth-membrane fraction. The incorporation of phosphate into both smooth- and rough-membrane fractions was approx. 8 pmol/mg of protein (mean of four experiments), or 1.7 x 10$^4$ d.p.m./mg of protein under the conditions described as standard in the Experimental section. It is noteworthy that when the range of concentration of ATP was extended to 20 mM (as compared with a concentration of 22 μM used in the standard assay), the patterns of phosphorylation of the smooth- and rough-membrane fractions remained essentially identical with those shown in tracks 3 and 8 respectively, although the total amount of phosphate incorporated was appreciably greater at the higher concentrations of ATP. An analysis of the effects of varying the concentration of Mg$^{2+}$ from 0 to 100 mM on the phosphorylation of the polypeptides of both smooth- and rough-membrane fractions is also shown in Fig. 1. In comparison with the control tracks (1 and 6, no Mg$^{2+}$ added), the phosphorylation of a large number of polypeptides of both smooth- (tracks 2–5) and rough- (tracks 7–10) membrane fractions is seen to be markedly stimulated by the addition of this cation. The most noticeable effect in both membrane fractions is the marked stimulation of the phosphorylation of a polypeptide of approx. 12 000 mol wt.; although stimulation of this component was evident at concentrations of Mg$^{2+}$ of 5 and 10 mM, the effect was even more pronounced (approx. 10-fold as estimated by counting of gel radioactivity) at 100 mM. Another noteworthy feature shown by this analysis is the marked stimulation of incorporation of radioactivity into the broad zone migrating near the front of the gel; again, the stimulation of incorporation of $^{32}$P into this zone was evident in both membrane fractions and was most marked (approx. 20-fold as estimated by counting of gel radioactivity).
Fig. 1. Radioautographic analysis of the effects of increasing Mg$^{2+}$ and Ca$^{2+}$ concentrations on the phosphorylation of polypeptides of microsomal-membrane fractions in vitro

Tracks 1–5, smooth-membrane fraction with 0 (i.e. no addition), 1, 5, 10 and 100 mM-Mg$^{2+}$ respectively; tracks 6–10, rough-membrane fraction with 0 (i.e. no addition), 1, 5, 10 and 100 mM-Mg$^{2+}$ respectively; tracks 11–14, smooth-membrane fraction with 0 (i.e. no addition), 10 μM and 1 and 10 mM-Ca$^{2+}$ respectively. The membrane fractions used were treated to remove ribosomes (stripped) before use. No Mg$^{2+}$ was added to the incubation mixtures in which the effects of Ca$^{2+}$ were tested. Results essentially identical with those shown for the smooth-membrane fraction were obtained for the rough-membrane fraction in the case of Ca$^{2+}$. Results similar to those shown for Mg$^{2+}$ and Ca$^{2+}$ were obtained in two other experiments of like design. The radioactive zone at the front (top) of the radioautogram corresponds to the $^{32}$P-containing lipid component(s). Phosphorylation of the polypeptide of approx. 12000 mol.wt. (indicated by the white dot in track 5) was particularly responsive to increasing Mg$^{2+}$ concentrations. Phosphorylation of the polypeptide of approx. 41000 mol.wt. (indicated by the white dot in track 3) was more prominent in the smooth-membrane fraction, whereas phosphorylation of the polypeptides of approx. 62000 and 35000 mol.wt. (indicated by the white dots in track 8) was more prominent in the rough-membrane fraction.

Radioactivity) at the highest concentration of Mg$^{2+}$ studied (100 mM). The nature of this zone is discussed below. Analysis of the effects of increasing the concentration of Ca$^{2+}$ (from 0 to 10 mM) on the phosphorylation of the smooth-membrane fraction is also shown in tracks 11–14; it is evident that addition of this cation exerted little effect on the pattern of phosphorylation. A similar lack of effect of addition of Ca$^{2+}$ on the pattern of phosphorylation of the rough membrane was also observed (results not shown). Essentially similar results to these shown in Fig. 1 were obtained from analyses of two other preparations of smooth- and rough-membrane fractions. No consistent differences in phosphorylation were observed between unfrozen and frozen membrane fractions in either the above
experiment or in those the results of which are described below.

The effects on the phosphorylation of microsomal-membrane polypeptides of substituting GTP for ATP were also examined. A typical analysis of experiments of this nature is shown in Fig. 2. The results shown were obtained from the total microsomal fraction (stripped of ribosomes); however, essentially similar results were found when smooth- and rough-membrane fractions were analysed separately. Track 1 shows the results of incubation with $[\gamma-32P]ATP$, under standard conditions; the results are generally similar to those shown in Fig. 1 (cf. track 8). Track 2 of Fig. 2 shows the results of adding an excess of non-radioactive ATP to the standard incubation mixture; the resultant lowering of the specific radioactivity of the ATP led to a marked diminution of the phosphorylation of all of the polypeptides, although appreciable incorporation of radioactivity into the component(s) migrating at the front of the gel was still evident. Track 3 shows the effects of adding excess non-radioactive GTP to the radioactive ATP; apart from a decrease in the phosphorylation of the polypeptide of approx. 12000 mol.wt. (that shown in Fig. 1 to be particularly responsive to addition of Mg$^{2+}$), little change in phosphorylation pattern is evident. Track 4 shows the results of incubation with $[\gamma-32P]GTP$, at the same concentration and specific radioactivity as that of the $[\gamma-32P]ATP$ used; in comparison with the results obtained with $[\gamma-32P]ATP$ (track 1), a marked diminution of total incorporation of $32P$ is evident, and the number of phosphopolypeptides detectable was considerably less than in the case with $[\gamma-32P]ATP$. A polypeptide of approx. 96000 mol.wt. (indicated by the white dot in track 4) was consistently labelled when $[\gamma-32P]GTP$ was used, but was scarcely or not at all labelled in the presence of $[\gamma-32P]ATP$. In addition, the labelling of the polypeptide of approx. 56000 mol.wt. (cf. Fig. 3) was relatively prominent. Track 5 shows the results of incubation with $[\gamma-32P]GTP$ in the presence of excess non-radioactive ATP. In comparison with the previous slot the phosphorylation pattern more closely resembled that produced by incubation with $[\gamma-32P]ATP$ (track 1), although the total incorporation was much less. In particular, labelling of the components of approx. 96000 and 56000 mol.wt. was considerably decreased (in comparison with slot 4) and phosphorylation of the component at the front of the gel was more marked. Track 6 shows the effects of adding excess non-radioactive GTP to the $[\gamma-32P]GTP$; as in the case of the corresponding analysis with $[\gamma-32P]ATP$ and non-radioactive ATP (track 2), the resultant lowering of the specific radioactivity of the nucleoside triphosphate markedly diminished the incorporation of $32P$ into all of the polypeptides. A noteworthy feature of these experiments was that whereas addition of the corresponding non-radioactive nucleoside triphosphate resulted in a marked diminution of incorporation of radioactivity (tracks 2 and 6), addition of the heterologous nucleoside triphosphate did not as markedly diminish the incor-

Fig. 2. Radioautographic analysis of the effects of adding excess non-radioactive ATP and GTP on the patterns of phosphorylation of total microsomal-membrane-fraction polypeptides produced by incubation with $[\gamma-32P]ATP$ or $[\gamma-32P]GTP$

The individual tracks show the patterns of phosphorylation of microsomal-membrane polypeptides produced by incubation with: track 1, $[\gamma-32P]ATP$; track 2, $[\gamma-32P]ATP$ in the presence of excess non-radioactive ATP; track 3, $[\gamma-32P]ATP$ in the presence of excess non-radioactive GTP; track 4, $[\gamma-32P]GTP$; track 5, $[\gamma-32P]GTP$ in the presence of excess non-radioactive ATP; track 6, $[\gamma-32P]GTP$ in the presence of non-radioactive GTP. Stripped total microsomal-membrane fractions were incubated in vitro with $[\gamma-32P]ATP$ or $[\gamma-32P]GTP$ under the standard conditions described in the Experimental section. A 1000-fold excess of non-radioactive ATP or GTP was added to each of the incubation mixtures, as indicated above. The slower migrating polypeptide (approx. 96000 mol.wt.) indicated by the white dot in track 4 was phosphorylated in the presence of GTP but not of ATP; the faster migrating polypeptide indicated by the white dot in this track had an approx. mol.wt. of 56000 and corresponds to the polypeptide shown in Fig. 3 whose phosphorylation was decreased by addition of cyclic AMP. Similar results to these shown here were obtained in two other experiments of like design.
poration of $^{32}$P into the individual polypeptides (tracks 3 and 5), although it did exert certain noteworthy effects.

The effect of addition of cyclic AMP on the phosphorylation of microsomal-membrane polypeptides by $[\gamma-^{32}\text{P}]$ATP was studied. For these experiments, the cyclic nucleotide in concentrations ranging from 0.01 to 100 $\mu$M was added to the standard incubation mixture. As shown in Fig. 3(a), addition of this compound at concentrations inclu-

![Fig. 3. Radioautographic analyses of the effects of addition of cyclic nucleotides on the phosphorylation in vitro of the polypeptides of the membranes of the microsomal fraction of mouse liver](image)

(a) Track 1, rough-membrane fraction, + cyclic AMP; track 2, rough-membrane fraction, − cyclic AMP; track 3, smooth-membrane fraction, − cyclic AMP; track 4, smooth-membrane fraction, + cyclic AMP. The conditions of phosphorylation were those referred to as standard in the Experimental section, except that cyclic AMP (4 $\mu$M) was added to the samples indicated above. Increased phosphorylation of the polypeptide indicated by the white dot in track 1 was not a consistent difference between the rough- and smooth-membrane fractions. Phosphorylation of the polypeptide indicated by the white dot in track 2 was consistently diminished by addition of cyclic AMP; this was the only consistent effect of cyclic AMP that was noted. Similar results to these shown were obtained in three other experiments of like design. (b) The individual tracks show the patterns of phosphorylation of the total microsomal-membrane fraction (stripped) produced by incubation with: track 1, $[\gamma-^{32}\text{P}]$ATP; track 2, $[\gamma-^{32}\text{P}]$ATP + cyclic GMP; track 3, $[\gamma-^{32}\text{P}]$GTP; track 4, $[\gamma-^{32}\text{P}]$GTP + cyclic AMP; track 5, $[\gamma-^{32}\text{P}]$GTP + cyclic GMP. The conditions of phosphorylation were those referred to as standard in the Experimental section, except that $[\gamma-^{32}\text{P}]$GTP was substituted for $[\gamma-^{32}\text{P}]$ATP in certain of the samples and cyclic GMP or cyclic AMP (each at 4 $\mu$M) was also added as specified above. Similar results to these were obtained in two other experiments of like design.
PHOSPHORYLATION OF POLYPEPTIDES OF MICROSOMAL MEMBRANES

Fig. 4. Analysis by radioautography of (a) the lipids extracted by acidified chloroform/methanol from $^{32}$P-labelled microsomal membranes and (b) the products of hydrolysis of fractions derived from the stripped total microsomal membrane labelled by phosphorylation in vitro

(a) 1, separation by t.l.c. of the inositol-containing phospholipids; 2, radioautogram of the separation shown in 1. The stripped total microsomal fraction was phosphorylated in vitro as described in the Experimental section. The fraction was next extracted with chloroform/methanol (three times) and then with acidified chloroform/methanol. Small amounts of non-radioactive inositol-containing lipids were added to the latter extract, which was then subjected to t.l.c. with chloroform/methanol/aq. 4M-NH$_3$ (9:7:2, by vol.) as the solvent system. The chromatogram was subjected to radioautography, the results of which are shown in 2. After this, the chromatogram was sprayed with the reagent of Vaskovsky & Kostetskya (1968) in order to reveal the migration of the phospholipids, as shown in 1. F, solvent front; O, origin; PI, DPI and TPI indicate the positions of 1-(3-in-phosphatidylinositol, 1-phosphatidylinositol 4-phosphate and 1-phosphatidylinositol 3,4-bisphosphate respectively. Essentially identical results were obtained in two other experiments of like design. (b) 3, Fraction extracted by acidified chloroform/methanol; 4, the insoluble residue left after extraction by acidified chloroform/methanol; 5, the total membrane fraction (not lipid-extracted). The stripped total microsomal fraction was phosphorylated in vitro. A portion of this fraction was then subjected to partial acid hydrolysis (see the Experimental section) and applied to channel 5. The remainder was first extracted with chloroform/methanol and then with acidified chloroform/methanol as described in the Experimental section. The latter extract was dried, subjected to partial acid hydrolysis and applied to channel 3. The insoluble non-lipid material remaining after acid lipid extraction was also subjected to partial acid hydrolysis and applied to channel 4. The products of hydrolysis of all three fractions were separated by electrophoresis and the electrophoreogram then covered with X-ray film. The migrations of $^{32}$P, phosphoserine (Ser-P) and phosphothreonine (Thr-P) were determined by the use of appropriate standards. Compound X is possibly inositol bisphosphate. The nature of the compound migrating near the origin in channel 4 is not known. Similar results to these were obtained in two other experiments of like design.

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To investigate this possibility, total microsomal fractions labelled in vitro were extracted with chloroform/methanol (2:1, v/v) and then with chloroform/methanol (2:1, v/v) containing 0.25% (v/v) constant-boiling HCl. Very little radioactivity (approx. 5%) was detected in the first solvent, whereas the second solvent extracted approx. 40% of the total radioactivity present in the membrane fraction (under the standard conditions of phosphorylation). The nature of the components present in the second extract was analysed by t.l.c. and subsequent radioautography. A typical radioautogram is shown in Fig. 4 (track 2); appropriate standards are shown for comparison in track 1. It is apparent that the major radioactive component corresponded in migration to 1-phosphatidylinositol 4-phosphate; a trace of radioactivity was evident in the area of the chromatogram corresponding to a standard of 1-phosphatidylinositol 3,4-bisphosphate and a somewhat greater amount of radioactivity was detectable in a compound migrating just between the above two lipids; the nature of this latter compound has not been established. Essentially similar results to those shown in Fig. 4(a) were obtained in two other experiments of the same design. The effects of extraction with acidified chloroform/methanol on the nature of the components present in hydrolysates of microsomal membrane fractions that had been phosphorylated in vitro were also investigated. A typical result (from one of three similar experiments) is shown in Fig. 4(b). Track 5 shows the components present in the hydrolysate of the non-extracted membrane preparation; compounds corresponding in migration to Pn, phosphoserine and phosphothreonine are evident, as is a compound (X) migrating ahead of P1. In contrast, analysis of the hydrolysate of the material extracted by acidified chloroform/methanol (track 3) revealed only the presence of compound X and P1. Analysis of the membrane fraction remaining after extraction by acidified chloroform/methanol revealed principally Pn, phosphoserine and phosphothreonine (track 4); compound X had already been substantially removed by the acid lipid extraction. On the basis of its apparently highly negative charge, we propose that compound X may represent inositol bisphosphate; however, we have not directly tested this possibility, because of lack of the appropriate standard.

Discussion

The above results show that a relatively large number (minimum of 17) of polypeptides of mouse liver rough- and smooth-microsomal-membrane fractions may be phosphorylated in vitro using [γ-32P]ATP as the phosphate donor. Only small differences (about three polypeptides) in the patterns of phosphorylation of the two types of microsomal membrane have been detected (cf. Fig. 1). The number of phosphorylated polypeptides resolved exceeds that reported (approx. three to five) in previous studies of the phosphorylation in vitro of rat liver microsomal membranes (Jergil & Ohlsson, 1974; Sharma et al., 1976; Lam & Kasper, 1979), but approximates to the numbers reported by Sharma et al. (1978); possible reasons for the superior resolution obtained by our laboratory have been cited previously (Sharma et al., 1978).

The present results on mouse liver microsomal-membrane fractions (cf. Fig. 1) are in agreement with those reported on corresponding rat liver fractions that the enzymes responsible for the endogenous phosphorylation are activated by Mg2+ but are not affected by Ca2+ (Jergil & Ohlsson, 1974; Sharma et al., 1976; Lam & Kasper, 1979). However, the resolution obtained here has permitted us to conclude that the phosphorylation of certain specific polypeptides is particularly responsive to increasing Mg2+ concentrations; the most dramatic example of this is the polypeptide of approx. 12000 mol.wt. (cf. Fig. 1), whose phosphorylation was increased approx. 10-fold at 100 mM-Mg2+. It is possible that Mg2+ affects phosphorylation not only by activating the protein kinase(s), but also by altering the conformation of the microsomal membrane to permit more facile phosphorylation of certain polypeptides.

In general, cyclic AMP did not affect the pattern of phosphorylation of the microsomal membranes produced by incubation with [γ-32P]ATP, except for a consistent decrease in the phosphorylation of one polypeptide of approx. 56 000 mol.wt. (cf. Fig. 3a). This effect was also seen when [γ-32P]GTP was used as the phosphate donor (cf. Fig. 3b). Whether this effect is due to a specific inhibition of phosphorylation or to an increase in dephosphorylation has not been determined. It has been reported previously (De Lorenzo & Greengard, 1973) that cyclic AMP may decrease the dephosphorylation of one polypeptide in toad bladder membrane by the latter mechanism. The studies of Sharma et al. (1976) and of Lam & Kasper (1979) failed to reveal any effect of cyclic AMP on the phosphorylation of specific microsomal-membrane polypeptides. In contrast, Jergil et al. (1976) have indicated that this nucleotide does increase the phosphorylation of certain polypeptides of rat liver microsomal-membrane fractions. A possible explanation for the inability of this and the two other studies cited above to find a stimulatory effect of cyclic AMP on the phosphorylation of microsomal-membrane polypeptides is afforded by studies by Sommarin & Jergil (1978) and Tan et al. (1979) in which the nature of the protein kinases present in microsomal membranes was characterized. In particular, the latter
workers have shown that at least two types of protein kinases are present in the microsomal-membrane fraction of rat liver. The first type was found to be tightly bound to the membrane, was not affected by cyclic AMP and appeared to be responsible for the phosphorylation of endogenous membrane proteins. The second type was loosely bound to the microsomal membrane, was affected by addition of cyclic AMP and acted on exogenous protein substrates. It appears possible that this second type of kinase may have been largely removed during the preparation of our membrane fractions. Our results have also shown that cyclic GMP, under the conditions tested, did not noticeably affect the phosphorylation of microsomal-membrane polypeptides by either ATP or GTP.

The ability of [γ-32P]GTP to phosphorylate microsomal-membrane polypeptides was also investigated. Our results revealed that use of this donor resulted in considerably less incorporation of 32P than did use of [γ-32P]ATP. The results of these experiments are complicated by the possibility that transfer of 32P from GTP to ATP may have occurred during incubation by exchange and transfer reactions catalysed by the sequential action of appropriate enzymes located in the microsomal membrane (cf. Kuo, 1974). However, independent of this possibility, it is noteworthy that the phosphorylation of one polypeptide of approx. 96 000 mol. wt. was observed when GTP was used, but not when ATP was used as the phosphate donor (cf. Figs. 2 and 3). Thus, although GTP is a less effective donor and despite the possibility that some of its effects may be mediated by phosphate transfer to ATP, it still appears to exert at least one specific effect on the phosphorylation of microsomal-membrane polypeptides.

An important point that has been clarified in the present study concerns the nature of the 32P-containing zone observed at the front of the SDS/polyacrylamide-gel electrophoretograms. This zone was noted in our previous study with rat liver microsomal membranes (Sharma et al., 1978) and has also been observed by Lam & Kasper (1979). Our results indicate that this zone is principally comprised of 1-phosphatidylinositol 4-phosphate. It has been shown previously that the kinase(s) converting 1-(3-sn-phosphatidylinositol) to 1-phosphatidylinositol 4-phosphate and this lipid to 1-phosphatidylinositol 3,4-bisphosphate are present in the microsomal fraction of rat liver (Harwood & Hawthorne, 1969). It is thus not surprising that our studies should have detected the incorporation of appreciable amounts of 32P into 1-phosphatidylinositol 4-phosphate. It would appear that the formation of this particular phospholipid should be suspected during studies in vitro of membrane phosphorylation using [γ-32P]ATP when a radio-active zone is detected at the front of SDS/polyacrylamide-gel electrophoretograms. It merits comment that incorporation of 32P into this compound may lead to appreciable overestimation of the amount of 32P incorporated into microsomal-membrane polypeptide fractions, if the membrane fractions are not extracted with a solvent capable of removing this lipid. A similar effect has been noted previously during studies of phosphorylation in vitro of the polypeptides of erythrocyte membranes; analysis by t.l.c. revealed the presence of appreciable incorporation of radioactivity into the complex phosphoinositides of this membrane (cf. Buckley, 1977).

Our overall results are in agreement with previous conclusions that the integral protein kinases of liver microsomal membranes are non-cyclic-nucleotide-dependent (Sharma et al., 1976; Lam & Kasper, 1979). Certain special features of these enzymes have been revealed in the present study: the relatively large number of polypeptide substrates, specific effects of cyclic AMP and of GTP on the phosphorylation of individual polypeptides, the marked effect of Mg2+ on the phosphorylation of certain polypeptides and the concomitant but presumably independent incorporation of 32P into 1-phosphatidylinositol 4-phosphate. Our previous study (Sharma et al., 1978) showed that many polypeptides of both types of microsomal membranes of rat and mouse liver may be phosphorylated in vitro, indicating that phosphorylation of these components occurs under physiological conditions. The central problem in this area would now appear to be the elucidation of the possible functional significance of the observation that many microsomal-membrane polypeptides are phosphorylated. Appropriate studies on individual microsomal-membrane proteins that appear to be phosphorylated should assist in this endeavour.

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