Electrophoretic Studies on Liver Endoplasmic Reticulum Membrane Polypeptides and on Their Phosphorylation in Vivo and in Vitro*

(Received for publication, August 29, 1977)

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to examine the polypeptide patterns of rat liver rough and smooth endoplasmic reticulum (ER) membrane fractions stripped of ribosomes. Approximately 67 polypeptides were resolved from the rough ER membrane fraction. The polypeptide pattern of the smooth ER membrane fraction was similar to that of the rough ER membrane fraction, but exhibited substantially lower amounts of some seven polypeptides. Three of these polypeptides, of apparent molecular weights 63,000, 65,000, and 87,000, were of particular interest, as they could not be ascribed to contamination of stripped rough ER membrane fractions by residual ribosomal polypeptides. Conditions of treatment with low concentrations of trypsin were established that markedly diminished the capacity of the stripped rough ER membrane fraction to bind ribosomes in vitro and that also effected a partial detachment of ribosomes from nonstripped rough ER membranes; the results of electrophoretic analyses of rough ER membrane fractions treated in these manners are described. Comparison of the polypeptide patterns of guinea pig, mouse, and rabbit liver ER membrane fractions with rat liver ER membrane fractions revealed considerable variations in the distributions of the polypeptides of 63,000, 65,000, and 87,000 molecular weight among the ER membrane fractions of these species. The combined results of these studies indicate that the polypeptide of 87,000 molecular weight, although particularly sensitive to attack by trypsin, is not involved in the binding of ribosomes to the rough ER membrane fraction. Studies by others (cf. Kreibich, G., Grebenau, R., Mok, W., Pereyra, B., Rodriguez-Boulan, E., and Sabatini, D. D. (1977) Fed. Proc. 36, 656) have implicated the polypeptides of 63,000 and 65,000 molecular weight in this process.

The patterns of phosphorylated polypeptides of rough and smooth ER membrane fractions of rat and mouse liver were also examined, using labeling in vivo with sodium $^{32}$P phosphate or in vitro with $\gamma$-$^{32}$P]ATP. Approximately 25 phosphorylated components were resolved by electrophoresis in the ER membrane fractions of both species. Evidence is presented that suggests that the great majority of these components are phosphopolypeptides. Differences were noted in the patterns of phosphorylation produced by in vivo and in vitro labeling; minor differences were also observed between the patterns of phosphorylation of the rough and smooth ER membrane fractions in either situation.

The overall results afford an indirect approach toward evaluating the possible involvement of specific rough ER membrane polypeptides in ribosome-binding and reveal that liver ER membranes contain a substantially greater number of phosphorylated polypeptides than previously reported.

Knowledge of various aspects of microsomal membrane structure and function has expanded in recent years (3). One consideration that is basic toward establishing an understanding of the structure of microsomal membranes is resolution and enumeration of their constituent polypeptides. Two previous studies of the polypeptides of endoplasmic reticulum membranes, one using slab gel (4) and the other disc gel (5) sodium dodecyl sulfate-polyacrylamide electrophoresis, have shown that rat liver rough ER and smooth ER membrane fractions contain a minimum of 32 to 33 polypeptides. In the present study we have re-investigated this aspect of microsomal membrane structure using a one-dimensional slab gel electrophoretic system that appears to offer a relatively superior separation, partly because of the length (17 cm) of the separating gel employed.

Various aspects of the biochemistry of the ribosome-membrane interaction have been reviewed (6, 7). Several recent reports have implicated specific polypeptides of the rough ER membrane in the process of ribosome binding. Sabatini and co-workers (8-10) have documented the involvement of rough ER membrane polypeptides of approximately 63,000 and 65,000 molecular weight, using a variety of experimental approaches. Ohlsson and Jergil (11) have suggested that cytochrome P-450 may play a role in ribosome binding. Fujita et al. (12) isolated a group of membrane polypeptides from
bound polyribosomes that had been released by treatment with Triton X-100 and indicated the involvement of polyribosomes of 108,000, 99,000, and 65,000 molecular weight in the membrane-rabosome interaction. A number of studies have shown that proteases may markedly diminish the capacity of RER membranes to bind ribosomes (cf. Ref. 6). Jothy et al. (13) used this approach and found that three RER membrane polyribosomes of 120,000, 93,000, and 36,000 molecular weight were digested by treatments with low concentrations of several proteases under conditions in which the capacity of the RER membrane to bind ribosomes was markedly decreased; the polyribosome of 36,000 appeared of particular interest as it had previously been reported to be present in RER but not in SER membranes (3). In order to help identity putative polypeptide components of the ribosome-binding site of the RER membrane, we have also used this latter approach by examining the effects on the polyribosome pattern of the RER membrane of treatment with low concentrations of trypsin.

Comorosan and Lugojan (14) provided some evidence that phosphoproteins may be involved in the process of ribosome binding. Subsequently, Jergil and Ohlsson (15) and Sharma et al. (16) have compared the patterns of phosphorylated polyribosomes of ER membranes of rat liver after incubation in vitro with \( \gamma^{13}P \)ATP. Both groups of workers described some of the properties of the microsomal protein kinase(s) catalysing this endogenous phosphorylation. In the present study we have compared the patterns of phosphorylated polyribosomes of RER and SER membranes produced by administration of sodium \([3'P]phosphate in vivo and by incubation with \( \gamma^{32}P \)ATP in vitro. Determination of the former is important in assessing the possible physiological significance of phosphorylation of ER membrane polyribosomes, because phosphorylation in vitro may be partly artifactual.

The results of this investigation significantly expand the number of polyribosomes previously reported in ER membranes, demonstrate that a substantial number of ER membrane polyribosomes may be phosphorylated both in vivo and in vitro, and afford an indirect approach toward evaluating whether specific polyribosomes of the RER membrane may be involved in the process of ribosome binding.

**Materials and Methods**

**Animals**

Female Wistar strain rats of 150 to 200 g body weight or female Swiss strain mice of approximately 20 g body weight were used in most experiments. Livers from four female albino guinea pigs (approximately 400 g body weight) and four female New Zealand white rabbits (approximately 2 kg body weight) were also obtained. All animals were starved for 16 h prior to death.

**Injection of Sodium \([3'P]phosphate**

A pilot experiment indicated that maximal incorporation of \( \gamma^{32}P \) into protein of the microsomal membrane occurred approximately 2 h after injection of sodium \([3'P]phosphate. Accordingly, to determine whether ER membrane polyribosomes were phosphorylated in vivo, four individual rats were injected on separate occasions with 5 ml of carrier-free sodium \([3'P]phosphate/100 g body weight. Similarly, four mice were injected with 5 ml of this isotope/20 g body weight. The animals were all killed 2 h after injection, and total microsomal and RER and SER membrane fractions were prepared as described below. The stripped RER and SER membrane fractions from rat liver were found to contain 1.1 to 1.4 \times 10^{4} dpm/mg of protein (range of values from four rats); the corresponding figures from the mouse liver ER membrane fractions were 2.7 to 3.0 \times 10^{4} dpm/mg of protein. Approximately 15% of these radioactivities were retained in the residual membrane fractions after three successive extractions of the stripped ER membrane fractions with chloroform:methanol (2:1, v/v).

**Preparation of ER Fractions**

### a. Total Microsomal Fractions

The livers were gently homogenized for 10 strokes with a Potter-Elvehjem homogenizer in ice-cold 0.2 M sucrose containing 0.05 M Tris/HCl (pH 7.4), 0.05 M KCl, 0.005 M MgCl\(_2\), and then centrifuged at 12,000 rpm (17,000 \( \times g_{\text{max}} \)) in the SS-34 rotor of a Sorvall centrifuge at 4°C for 10 min with the brake on. The supernatant (S-2 fractions) were gently removed using Pasteur pipettes and subjected to centrifugation at 30,000 rpm (150,000 \( \times g_{\text{max}} \)) in the A-110 rotor of an International Equipment Co. (I.E.C.) ultracentrifuge for 1 h. The resulting supernatants (S-3 fractions) were then carefully decanted and in some cases (e.g. in experiments where \( ^{32}P \) was administered) saved for further analyses. The pelleted total microsomal fractions were washed once gently rehomogenization in 25 ml of 0.2 M STKM buffer and rehomogenized as just described.

### b. RER and SER Membrane Fractions

These were prepared by two methods initially. In Method A, preparation was from the S-2 fraction by adjusting it to 1.33 M STKM buffer by gradual addition of 2.0 M STKM buffer and in turn covered by a zone of 0.2 M STKM buffer. Centrifugation was then performed at 105,000 \( \times g_{\text{max}} \) for 15 h again using the A-110 rotor of an I.E.C. ultracentrifuge. RER and SER fractions were collected from their respective positions in the centrifuge tubes as previously described (5). In Method B, preparation was from the pelleted total microsomal fractions. These were first resuspended in a small volume of 0.2 M STKM buffer by gentle homogenization and then adjusted to 1.33 M STKM buffer as in Method A. Further procedures were as described for Method A. Electrophoretic analyses of the RER and SER fractions obtained by Methods A and B revealed that the polypeptide pattern of the RER membrane fractions obtained by these two methods were identical and that the polypeptide patterns of the RER membrane fractions were also identical. Accordingly, for reasons of convenience, Method B was used routinely in the studies described here.

The SER fractions were further purified routinely as follows. The pelleted fractions obtained by Method B were rehomogenized in 1.23 M STKM buffer and layered between 2.0 and 0.2 M STKM buffer as described in Method A. Centrifugation was then performed as in Method A but for a period of 4 h. The fraction floating at the top of the 1.23 M STKM buffer zone was collected and pelleted as described for preparation of the total microsomal fraction.

### Removal (stripping) of ribosomal components from total microsomal and ER membrane fractions was performed using a reagent comprised of 0.26 M sucrose, 0.08 M sodium citrate, 0.01 M sodium pyrophosphate, 0.01 M sodium phosphate (pH 7.4) (19) as described previously (5); generally, a total of three treatments with this medium was used. This procedure has been shown to reduce the RNA/protein ratio of the RER fraction to approximately 7% of its original value (5). In some experiments total microsomal and ER membrane fractions were stripped using the poronycin/ECI method (20).

For purposes of electrophoretic comparison with ribosomal polyribosomes present in ER membrane fractions, large and small ribosomal subunits of rat liver were prepared as described previously (20).

**Sonication of Membrane Fractions**

Approximately 0.5-mg aliquots (based on protein) of both nonlabeled and \( ^{32}P \)-labeled ER membrane fractions were suspended in 5 ml of distilled water and subjected to vigorous sonication as described previously (5). The residual membrane fractions were pelleted by ultracentrifugation as described for preparation of the total microsomal fraction and subjected to electrophoresis. In some cases, the supernatants remaining after ultracentrifugation were concentrated by lyophilization and also analyzed by electrophoresis.

### Ribosome-binding Assay

The capacity of stripped RER membrane fractions and of these fractions treated with low amounts of trypsin (see below) to bind ribosomes was assayed as described by Rolleston (20).

### In Vitro Phosphorylation

This was performed using the following mixture based on that described by Jergil and Ohlsson (15): (1) 1 mg of membrane protein,
cases, cyclic AMP (5 nmol) was included. Also, as a control, in dilution of the labeled ATP, this resulted in a lower specific activity of the membrane fractions and precluded their analysis by radioautography. When fractions were to be stripped of ribosomes, stripping medium was repeated twice more. For ethanolation extraction, the membranes were first precipitated with 3 ml of ice-cold 10% trichloroacetic acid and the resulting precipitate pelleted by centrifugation as just described. The precipitate was washed twice with ice-cold trichloroacetic acid and then extracted twice with 5 ml of ethanol/ether. The extracted pellets from both of these procedures were then taken up in the solubilizing medium used for electrophoresis.

Detection of Phosphoserine and Phosphothreonine

Stripped samples (about 2 mg of protein) of 3P-labeled RER and SER membranes were extracted with chloroform/methanol (2:1, v/v) and ether/ether (1:2, v/v). In each case, 500 μg of membrane protein were suspended in 50 μl of 0.2 M STKM buffer. For the extraction with the first solvent mixture, 3 ml of chloroform/methanol were added followed by vigorous shaking for 5 min. The resulting suspension was then centrifuged at 17,000 × g for 15 min. The supernatant was discarded and the entire procedure repeated twice more. The resulting suspensions were then precipitated with 3 ml of ice-cold 10% trichloroacetic acid and the resulting precipitate pelleted by centrifugation as just described. The precipitate was washed twice with ice-cold trichloroacetic acid and then extracted twice with 5 ml of ethanol/ether. The extracted pellets from both of these procedures were then taken up in the solubilizing medium used for electrophoresis.

Determination of Radioactivity

All radioactive determinations were performed using a Mark 1 liquid scintillation counter. Membrane samples were processed for analysis by either of two methods. In the first, 10 μl of membrane suspension (30 mg of protein/ml) were added 0.1 ml of water and 1.0 ml of NCS (Nuclear Chicago) solubilizer; the resulting mixture was agitated vigorously for several minutes and then allowed to stand at 22°C for 18 h. Alternatively, aliquots of membrane fractions were applied to filter paper discs which were then immersed in ice-cold 10% trichloroacetic acid for 20 min. The filter papers were allowed to dry at room temperature and placed in glass scintillation vials. Then 15 ml of a toluene-based liquid scintillation fluor were added to each type of processed sample and their radioactivity determined. Corrections for quenching were made by the channels ratio method when necessary.

RESULTS

Fig. 1 shows a comparison of the electrophoretic patterns (10% gel) of the polypeptides of the large ribosomal subunit (channels 1 and 2), the small ribosomal subunit (channels 3 and 4), and the stripped RER membrane fraction (channel 5). The ribosomal subunits were analyzed at two different amounts—75 μg of protein (channels 1 and 3) and also diluted to an amount approximating that at which they occur in unstripped RER membranes (channels 2 and 4). This system resolved approximately 24 polypeptides from the large ribosomal subunit and 15 from the small subunit. Additional components of both subunits were resolved in 12.5% gels (not shown here). The polypeptides of the stripped RER membrane were enumerated in subsequently presented electrophoretograms, when their pattern is compared with that of the SER membrane fraction. The principal feature illustrated by this figure is that whereas the ribosomal polypeptides are predominately fast migrating and of molecular weight less than 32,000, the polypeptides of the RER membrane are distributed across the whole length of the gel, varying in molecular weight from over 94,000 to under 25,000. The above information was important for the interpretation of subsequent electrophoretograms of stripped and unstripped membrane fractions; individual polypeptides removed by stripping could be
In vitro to approximately 15% of a control untreated sample. Fraction (channel 2). Treatment of the stripped SER membrane subunit; 3 and 4, small ribosomal subunit; 5, stripped RER. Approximately 75 pg of protein were applied in each of channels 1, 3, and 5 and 20 pg in each of channels 2 and 4. The numbers in the righthand margin represent the positions of marker proteins with molecular weights of 23,000, 62,000, 67,000, and 94,000.

Fig. 2 (right). Electrophoretic comparison (10% gel) of rat liver SER and RER fractions and of the effects of treatment with a low concentration of trypsin on these fractions. 1, SER stripped, then treated with trypsin; 2, SER stripped; 3, SER unstripped, treated with trypsin; 4, SER unstripped; 5, RER unstripped; 6, RER stripped, then treated with trypsin; 7, RER stripped; 8, RER unstripped, treated with trypsin. The numbers in the righthand margin represent the positions of marker proteins of known molecular weight (cf. Fig. 1). PB refers to the position of the phenobarbital-responsive species of cytochrome P-450 (Ref. 22). The bands indicated by the black dots along both sides of channel 7 were present in higher amounts in the stripped RER fraction than in the stripped SER fraction (channel 2). Treatment of the stripped RER membrane with the low concentration of trypsin used here (see "Materials and Methods") reduced the capacity of this membrane to bind ribosomes in vitro to approximately 15% of a control untreated sample.

Fig. 2 shows a comparison of the polypeptides of rat liver SER and RER fractions analyzed in a 10% gel. Channels 2 and 4 show the patterns of stripped and unstripped SER fractions, respectively; a number of fast migrating components, corresponding to ribosomal polypeptides, are seen to be removed by stripping. Comparison of the stripped SER (channel 2) and stripped RER (channel 3) fractions again reveals the general similarity of their polypeptide patterns. However, components of approximately 19,000, 20,000, 33,000, 40,000, 63,000, 65,000, and 87,000 molecular weight are seen to be more prominent in the stripped RER fraction, whereas the SER fraction exhibits one more prominent polypeptide of approximately 32,000 molecular weight. The differences of polypeptide pattern between SER and RER fractions shown in this figure and in Fig. 2 are consistent, as they have been observed in six different preparations of SER and RER membrane fractions. The effects of treatment of the unstripped RER fraction with a high concentration of trypsin (30 min) (see "Materials and Methods") are shown in channel 6; considerable modification of the electrophoretic pattern is evident, in comparison with the pattern exhibited by an appropriate control fraction (channel 5) incubated under the same conditions but in the absence of trypsin. In results not shown here, incubation with a high concentration of trypsin for 60 min was found to virtually eliminate all of the polypeptide bands resolved from both the SER and RER membrane fractions. In contrast, incubation of RER and SER membrane fractions with a low concentration of trypsin (see below) resulted in quite selective proteolysis of only certain polypeptides. Incubation of the unstripped RER fraction with RNase did not alter its pattern (channel 7). The polypeptide pattern of the stripped total microsomal fraction is also shown for comparison (channel 8); the pattern of this fraction exhibits all of the components seen in the stripped RER and SER fractions. Effects of trypsin and RNase on the pattern of this fraction are also shown in channels 10 and 11, respectively. Again, trypsin produced a marked alteration of pattern as compared with an appropriate control sample (channel 9), whereas RNase had no evident effect. The use of 15% gels did not improve the resolution of the low molecular weight polypeptides achieved in 12.5% gels. It was also noted that the
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**Fig. 3.** Electrophoretic and radioautographic comparisons of various rat liver ER fractions labeled in vivo with $^{32}$P. A, electropherogram (12.5% gel): 1, SER unstripped; 2, SER stripped; 3, RER stripped; 4, RER unstripped; 5, RER unstripped, control for trypsin incubation; 6, RER unstripped, incubated with trypsin; 7, RER unstripped, incubated with RNase; 8, total microsomal fraction stripped; 9, total microsomal fraction stripped, control for trypsin incubation; 10, total microsomal fraction stripped, incubated with trypsin; 11, total microsomal fraction stripped, incubated with RNase; 12, sample of trypsin and RNase; 13, molecular weight markers; 14, H chain of human IgG (52,000). B, radioautogram of the above electropherogram. The samples are in the same order as indicated above except that samples 12 to 14 have been omitted.

The molecular weight markers shown in channel 13 are cytochrome c (12,000), myoglobin (17,000), chymotrypsinogen A (25,000), rat serum albumin (67,000), and rabbit muscle phosphorylase a (94,000).

The band indicated by black dots in channel 1 (A) was present in higher amount in both SER fractions (also see channel 2) than in the stripped RER fraction. The bands indicated in channel 3 were present in higher amount in the stripped RER fraction than in the stripped SER fraction. The two radioactive bands indicated in channel 3 (B) were more prominent in the stripped RER fraction than in the stripped SER fraction. The fast migrating radioactive bands indicated in channel 4 were removed from the RER fraction by stripping.

Resolution afforded by 5 to 15% gradient gels was inferior to the combined resolution provided by the sequential use of 10 and 12.5% gels.

For ease of comparison with Fig. 3A, it is appropriate to discuss the radioautographic analysis of the same gel shown in Fig. 3B at this stage. Approximately 20 radioactive bands are seen in the unstripped SER fraction (channel 1); stripping did not affect this pattern (channel 2). The radioactive patterns of the stripped and unstripped RER fractions are seen in channels 3 and 4; a number of relatively fast migrating components, corresponding to ribosomal polypeptides, are seen to be removed by stripping. Comparison of the stripped RER (channel 3) and stripped SER (channel 2) fractions reveals that the principal differences in radioactive patterns are the presence in the former of two more prominent bands of approximately 11,000 and 33,000 molecular weight. Treatment of the unstripped RER fraction with a high concentration of trypsin (30 min) (channel 6) resulted in a marked diminution or elimination of most of the radioactive bands; treatment with the same amount of trypsin for 60 min (results not shown here) effected disappearance of all of the original radioactive components migrating behind the gel front. In contrast, incubation with RNase (channel 7) produced little effect on the radioactive pattern of this fraction. The radioactive pattern of the stripped total microsomal fraction (channel 8) closely resembled that of the stripped RER fraction (channel 3). The results of incubation of this fraction with trypsin (channel 10) and RNase (channel 11) were identical with those described for the RER membrane fraction.

Treatment of stripped RER membranes with various concentrations of trypsin was investigated in order to determine a minimal concentration that would markedly affect the ability of this fraction to bind ribosomes in vitro. Incubation at a trypsin:membrane protein ratio of 1:5000 (see "Materials..."
and Methods”) was found to reduce the capacity of stripped RER membranes to bind ribosomes to 12, 15 and 20% of control values in three separate experiments. Incubation in buffer without trypsin or addition of soybean trypsin inhibitor alone did not affect the capacity of stripped RER membranes to bind ribosomes. Accordingly, the effects of treatment with this concentration of trypsin on the electrophoretic patterns of both stripped and unstripped RER and SER membrane fractions were investigated. Results of this study are shown in Fig. 2. The pattern of the control stripped RER fraction (channel 7) has been described previously. Incubation with trypsin (channel 6) is seen to produce quite minor changes in the pattern of this fraction; the most prominent changes are reductions in the intensities of those zones that are present in greater amounts in the stripped RER fraction than in the stripped SER fraction (comparison of channels 7 and 2), although the intensities of several other bands are also seen to be diminished. Perhaps the most striking change is the virtual disappearance of the band of molecular weight 87,000 with the appearance of a new zone just ahead of it. The polypeptides of 63,000 and 65,000 (previously implicated by Kreibich et al. (8-10) in ribosome binding) appear to be only partly affected by this treatment (comparison of channels 5 to 7). The unstripped RER fraction (channel 5) was also treated similarly with trypsin (channel 8); the migration of a number of the fast migrating ribosomal polypeptides is seen to be altered by this treatment as is the migration of the polypeptide of 87,000. Essentially similar results were obtained when stripped RER membrane fractions treated with trypsin were analyzed in 12.5% gels, i.e. almost complete apparent conversion of the polypeptide of 87,000 to a slightly faster migrating zone, a moderate reduction in the staining intensities of the polypeptides of 63,000 and 65,000 and evidence of proteolytic cleavage of four relatively low molecular weight polypeptides.

To obtain further information regarding the possible involvement of specific RER membrane polypeptides in ribosome binding, unstripped RER fractions were treated with an intermediate concentration of trypsin (see “Materials and Methods”) that resulted in partial conversion of the RER fraction to a lighter floating SER fraction. (In each of three separate experiments, approximately 30% of the initial RER fraction was converted.) Fig. 4 is an electrophoretic analysis of one of three such experiments, all yielding similar results. Channels 1, 2, and 3 show the patterns of control fractions of stripped SER, unstripped RER, and stripped RER, respectively. Channel 4 shows the pattern of a sample of stripped RER treated with the same concentration of trypsin as used to effect partial conversion of unstripped RER to a SER fraction. Channels 5, 6, and 7, respectively, show the patterns of the light fraction (unstripped), the heavy fraction (unstripped), and the heavy fraction (stripped) obtained after ultracentrifugal separation of the trypsin-treated RER fraction. The principal feature of interest shown by this gel is that proteolysis of the band of 87,000 molecular weight was evident in all of the fractions treated with trypsin; of particular significance was the observation that this band had been digested even in the residual RER (heavy) fraction obtained after ultracentrifugation (channels 6 and 7). Also of interest is the observation that the bands of 63,000 and 65,000 were not markedly affected by treatment with trypsin in either the light or heavy fractions obtained after ultracentrifugal separation. Electron microscopic examination of the light and heavy fractions recovered from these experiments revealed that the light membrane fraction was essentially free of ribosomes, whereas the heavy fraction was comprised principally of apparently normal looking membrane vesicles studied with ribosomes along with some free ribosomes.

In order to assess whether the polypeptides of 63,000, 65,000, and 87,000 molecular weight were consistently increased in amount in RER fractions as compared with SER fractions, the polypeptide patterns of stripped liver ER membrane fractions from the livers of several other species were compared with those of rat liver as shown in Fig. 5. The patterns of rat RER and SER fractions are shown in channels 4 and 5, respectively; the polypeptides of 63,000, 65,000, and 87,000 molecular weight are seen to be more prominent in the RER fraction than in the SER fraction, in confirmation of the results shown in Figs. 2 and 3. Both the polypeptides of 63,000 and 65,000 appeared more marked in the RER fraction of mouse liver (channel 1) as compared with the SER fraction (channel 2); the polypeptide of 87,000 was not prominent in either fraction. Examination of the guinea pig fractions revealed that the polypeptide of 65,000 was prominent in the RER fraction (channel 7), whereas the polypeptides of 63,000 and 87,000 were barely visible in either fraction (channels 6 and 7). In the rabbit liver fractions, polypeptides of 64,000 and 87,000 molecular weight were more prominent in the RER fraction (channel 6), whereas bands of 63,000 and 65,000 appeared to

![Fig. 4 (left). Electrophoretic analysis (10% gel) of treatment of the unstripped RER fraction with a concentration of trypsin causing partial detachment of ribosomes (see "Materials and Methods"). 1, SER stripped; 2, RER unstripped; 3, RER stripped; 4, RER stripped plus trypsin; 5, the light fraction (unstripped) obtained by centrifugation after treatment of unstripped RER with trypsin; 6, the heavy fraction (unstripped) obtained after treatment of unstripped RER with trypsin; 7, the heavy fraction (stripped) obtained by centrifugation after treatment of unstripped RER with trypsin. The polypeptide of 87,000 molecular weight is indicated by black dots in channel 3. The apparent product of proteolysis of this polypeptide is indicated in channel 5 as are the two polypeptides of 63,000 and 65,000 molecular weight.](http://www.jbc.org/DownloadedFrom)
be absent in both fractions (channels 8 and 9). Relatively minor differences in polypeptide pattern between the RER and SER fractions of each species were observable, as were more conspicuous differences in pattern between the corresponding ER fractions of each species. However, no absolutely consistent differences in pattern between the RER and SER fractions of all the species were observed.

Evidence has already been presented (Fig. 3B) for the presence in total microsomal, RER, and SER liver membrane fractions of some 20 radioactive components following the in vivo administration of sodium $^{32}$P phosphate to rats. An electrophoretic and radioautographic comparison of the patterns of the in vivo $^{32}$P-labeled components of the SER and RER membrane fractions of rat liver with those of some other subcellular fractions from this organ is shown in the right hand portion of Fig. 6, A and B. Both the polypeptide and radioactive patterns of the S-3 fraction (channel 10), the mitochondrial fraction (channel 11), and the total homogenate (channel 12) are seen to differ quite markedly from each other and from the unstripped and stripped SER (channels 6 and 7) and RER (channels 9 and 8, respectively) fractions. It was also observed that serum taken from rats injected with $^{32}$P exhibited several $^{32}$P-containing components, only one of which of approximately 60,000 molecular weight is visible in channel 13. Fig. 6B is of further interest in that it also shows a comparison of the $^{32}$P-containing components of SER and RER fractions of rat liver following incubation in vitro with [γ-$^{32}$P]ATP. The fractions were subjected to phosphorylation in vitro and then either stripped or not stripped. The polypeptide patterns of the fractions analyzed are shown in channels 1 to 4 of Fig. 6A for comparison, and are essentially similar to those presented in Fig. 3A. The radioactive patterns of unstripped and stripped SER are shown in channels 1 and 2 of Fig. 6B; no differences in pattern between these SER fractions were noted. The radioactive patterns of stripped and unstripped RER fractions are shown in channels 3 and 4; stripping of this fraction was found to markedly reduce or eliminate a number of the radioactive components corresponding to ribosomal polypeptides. (Summation of results from four different experiments indicated that nine radioactive

![Figure 6](http://www.jbc.org/)

**Fig. 6.** Electrophoretic and radioautographic comparison of various rat liver fractions labeled in vitro or in vivo. A, electrophorogram (12.5% gel): 1, SER unstripped labeled in vitro; 2, SER labeled in vitro, then stripped; 3, RER labeled in vitro, then stripped; 4, RER labeled in vitro, not stripped; 5, molecular weight markers; 6, SER labeled in vitro, not stripped; 7, SER labeled in vitro, then stripped; 8, RER labeled in vitro, then stripped; 9, RER labeled in vitro, not stripped; 10, S-3 fraction labeled in vitro; 11, mitochondrial fraction labeled in vitro; 12, total homogenate labeled in vitro; 13, rat serum, labeled in vitro. B, radioautogram of the above electrophorogram. The samples are in the same order as indicated above. The radioactive band indicated in channel 1 was more prominent in the stripped SER fraction labeled in vitro than in the stripped RER fraction. Conversely, the two bands indicated in channel 3 were more prominent in the latter fraction. The band indicated in channel 6 labeled in vitro but not in vivo. The bands indicated in channel 8 were more prominent in the stripped RER fraction (labeled in vivo) than in the stripped SER fraction. The molecular weight markers used in this gel are the same as those shown in channels 13 and 14 of Fig. 3A.
ribosomal components were removed from the RER fraction by stripping.) The radioautographic patterns of stripped SER and stripped RER are seen to generally resemble one another, but three consistent differences were observable; two components (of 11,000 and 23,000 molecular weight) in the RER (channel 3) were labeled more prominently and one component (28,000) in the SER (channel 1) was labeled more heavily. Comparison of the radioactive patterns produced by in vivo and in vitro phosphorylation was of interest. Although different amounts of protein-bound radioactivity were applied in this experiment (approximately 2 times more in the case of the in vitro labeled samples), it is evident that there are appreciable quantitative and qualitative differences between the patterns produced by the two methods of phosphorylation. For instance, the band of approximately 17,000 molecular weight was heavily labeled in vitro but barely labeled in vivo. Conversely, the band of approximately 26,000 molecular weight (indicated in channel 6), which was heavily labeled in vivo, did not appear to label in vitro. Of interest also, is the observation that bands of molecular weight 52,000 and 54,000, previously shown to correspond in migration to cytochromes P-450 and P-450, species (22), were radioactive in both types of labeling situation. The patterns of phosphorylation produced by both in vivo and in vitro labeling with $^3P$ were consistent in that essentially the same patterns were observed in each of four separate experiments involving both types of labeling. When [alpha-$^3P$]ATP was substituted for [gamma-$^3P$]ATP in the reaction mixture for in vitro phosphorylation, no radioactive bands were detected following electrophoresis. Cyclic AMP at the concentration tested did not result in any detectable alteration of the pattern of phosphorylation of either ER membrane. The patterns of phosphorylation of mouse liver ER membrane fractions were also investigated electrophoretically (results not shown here). Administration of sodium [$^3P$]phosphate in vivo resulted in complex patterns of phosphorylated bands in both stripped RER and SER fractions, which were very similar to those described for the corresponding rat liver fractions. As noted for the rat liver fractions (Fig. 3B), two additional $^3P$-labeled polypeptides of 11,000 and 33,000 molecular weight were detected in the stripped RER fraction. The patterns of $^3P$-containing polypeptides of the mouse liver ER membrane fractions produced by in vitro phosphorylation also closely resembled that of the corresponding rat liver fractions, with two additional polypeptides of 11,000 and 23,000 molecular weight being noted in the stripped RER fraction and one of 28,000 molecular weight in the stripped SER fraction. It was also observed that, in similar fashion to the rat liver fractions, the polypeptide of 26,000 molecular weight was phosphorylated in vivo but not in vitro in both stripped RER and SER membrane fractions.

Stripped total microsomal membrane fractions from rat and mouse liver, labeled in vivo or in vitro, were subjected to various treatments in order to help characterize the nature of the $^3P$-containing bands detected by electrophoresis. The results of treatment of in vivo labeled rat liver samples with trypsin and RNase have already been described; similar results were observed with mouse liver fractions. Treatment with DNase of the total microsomal fractions labeled in vivo was found not to affect the pattern of radioactive bands. Treatment of in vitro labeled total microsomal fractions with a high concentration of trypsin resulted in almost complete elimination of all the radioactive components migrating in the gel, whereas RNase and DNase treatments did not affect the observed patterns. The stripped total microsomal fractions labeled in vivo were subjected to vigorous sonication in order to determine whether any of the phosphorylated bands represented loosely bound or perhaps intravesicular components; however, no change of pattern from those of untreated samples was noted. The stripped total microsomal fractions were also precipitated with 10% trichloroacetic acid and subsequently extracted with chloroform:methanol (2:1, v/v) or ethanolether (1:2, v/v). Neither of these two treatments affected the patterns of $^3P$-containing bands in the gels, but both markedly diminished the intense radioactive zones noted at the front of the gels (cf. Fig. 6B), particularly in the case of the in vivo labeled samples. Acid hydrolysis of lipid-extracted total microsomal fractions and subsequent analysis by high voltage electrophoresis revealed the presence of both radioactive phosphoserine and phosphothreonine in the hydrolysates; of the recovered radioactivities, approximately 15% was present in these two compounds, some 10% in two unidentified $^3P$-containing compounds and the remainder in free inorganic phosphate.

**DISCUSSION**

The results of this study, resolving some 67 and 60 polypeptides from RER and SER membranes, respectively, significantly expand the number of polypeptides detected in preparations of microsomal membranes stripped of ribosomes. Kreibich and Sabatini (4) reported the presence of approximately 33 polypeptides in RER and SER membranes. Bailey et al. (5) described a similar number of polypeptides in these membranes. Perhaps more intriguing is the present demonstration of several consistent differences between the polypeptide profiles of RER and SER membranes. Kreibich and Sabatini (4) found that the polypeptide profiles of these two membranes were identical. Bailey et al. (5) observed that the patterns of these two membranes were almost identical; however, the RER membrane fraction was found to contain one additional polypeptide of approximately 36,000 molecular weight. It was speculated that this particular polypeptide might be implicated in the process of ribosome-binding by the RER membrane. Comparison of the separation of Bailey et al. (5) (achieved using long SDS-polyacrylamide disc gels) with the present separations indicates that the single polypeptide of approximately 36,000 molecular weight reported to be additional in the RER membrane resolves in the present electrophoretic system into several minor components; the other differences between RER and SER membranes noted here were not previously observed because of the inferior resolving power of the disc gels used. More recently, Sabatini and his colleagues (8-10) have reported the presence of two polypeptides of approximately 63,000 and 65,000 molecular weight in the RER membrane that were barely detectable in the SER membrane. Two polypeptides of similar molecular weights were also observed in the present study to be substantially higher in amount in the RER membrane. It should be commented that the complexity of the polypeptide patterns resolved in this study has in one experimental sense proven disadvantageous, as we have not yet found it possible to obtain from the stained gels densitometric analyses that match the resolutions observed with the naked eye. For this reason, the description of electrophoretograms throughout this manuscript has been essentially qualitative.

Ribosomal and intravesicular polypeptides may both contribute to the electrophoretic patterns of microsomal membrane fractions (4). The relative freedom of the microsomal
membrane fractions analyzed here from contamination by ribosomal polypeptides is attested to by the disappearance or marked reduction following the rigorous stripping procedure used here of the fast migrating polypeptides corresponding in migration to bands resolved from purified ribosomal subunits. As monitored by electrophoresis (results not shown here), we have found the puromycin/KCl method to be less effective in removing the faster migrating ribosomal polypeptides than the pyrophosphate/citrate/phosphate reagent generally used in this study. The close similarity of the patterns of polypeptides of the stripped RER and unstripped SER membranes also supports the relative efficiency of the stripping procedure used. Moreover, the minimal effect of stripping on the polypeptide pattern of the SER fraction indicates that few if any "peripheral" membrane polypeptides were removed by the stripping procedure. The almost complete disappearance of a number of fast migrating phosphorylated polypeptides from the RER membrane on stripping further attests to the efficiency of stripping in removing ribosomal polypeptides. It is, however, difficult to exclude the possibility that a small number of the additional polypeptides noted in the RER membrane may represent very tightly bound ribosomal polypeptides; this possibility is unlikely to explain the differences in polypeptide patterns noted of over 82,000 molecular weight, as no prominent ribosomal polypeptides of over this molecular weight were noted.

The relative freedom of the RER membrane fraction from intravesicular polypeptides is principally evidenced by the lack of any prominent band in the region of rat serum albumin, the principal intravesicular protein of rat liver (cf. Ref. 4). (Prealbumin and albumin do not separate in the electrophoretic systems used in this study.) Vigorous sonication followed by centrifugation did not alter significantly the polypeptide patterns of the RER and SER membrane fractions (results not shown here), although the supernatant fractions were enriched in several components (including a band corresponding in migration to serum albumin) that were barely detectable in the nonsonicated membrane fractions. These findings confirm our previous observation that intravesicular polypeptides do not contribute significantly to the polypeptide patterns of the ER membrane fractions (5). This finding in no way denies the existence of intravesicular polypeptides (4), but only stresses that they do not contribute significantly to the polypeptide patterns as visualized by chemical staining. Indeed, in recent studies utilizing the intraperitoneal injection of high specific activity L-3H]methionine into rats and mice, we have detected the presence in the intravesicular compartment of some 15 radioactive polypeptides, the majority of which are precipitable by antiserum raised to whole sera, but do not correspond directly in electrophoretic migration to the polypeptides resolved here.2

2 M. Behar-Bannelier, R. N. Sharma, and R. K. Murray, manuscript in preparation.

A major task for the future will be the allotment of specific functions to the many bands detected in SDS-polyacrylamide electrophoretograms of ER membranes. At present, the only bands that we have identified are the phenobarbital-inducible species of cytochrome P-450 (cf. Fig. 2) and the 3-methylcholanthrene-inducible species of cytochrome P-450, (cf. Ref. 22).

The results of the experiments in which the electrophoretic patterns of trypsin-treated membranes (treated with a low but sufficient amount of this enzyme to markedly diminish the capacity of this membrane to bind ribosomes in vitro) were analyzed are of interest. Initial results from these experiments particularly attracted our attention to the polypeptide of approximately 87,000 molecular weight, present in significantly higher amounts in RER than SER membranes and whose electrophoretic migration was perceptibly affected by treatment with the low amount of trypsin used (cf. Fig. 2, channel 8). However, results from experiments in which the RER membrane was partly converted to SER by incubation with trypsin were revealing; these results showed that in the fraction of the RER membrane that had not been converted to SER by treatment with trypsin, this particular polypeptide had nevertheless been digested by the enzyme to approximately the same extent as in the fraction that had been converted to SER (Fig. 4, comparison of channels 5 to 7). It was thus concluded from this observation that digestion of this polypeptide was not necessarily accompanied by ribosome detachment. It should be noted that several relatively low molecular weight polypeptides (between 25,000 and 40,000 molecular weight) (comparison of Figs. 2 and 4, channels 6 and 7, respectively) were affected by trypsin in the experiments in which in vitro binding of ribosomes to membranes was monitored and in the experiments in which RER membranes were partially converted to SER membranes by treatment with this protease. Another revealing observation regarding the polypeptide of 87,000 was that it was not observed to be increased in amount in the RER fractions of guinea pig, mouse, and rabbit livers. Of interest also were the relative susceptibilities to trypsin of the polypeptides of 63,000 and 65,000 molecular weight, as polypeptides of these molecular weights have been implicated in the process of ribosome binding by rat liver ER membranes (8--10). Our observations indicated that these polypeptides were not remarkably susceptible to trypsin digestion, and in particular that they did not appear to have been appreciably digested by this enzyme in experiments where trypsin was used to reduce the capacity of the RER membrane to bind ribosomes in vitro or in the experiments where trypsin was used to convert RER to SER membranes. Kreibich et al. (8--10) have apparently found these polypeptides to be degraded by trypsin under conditions in which the capacity of RER membranes to bind ribosomes in vitro was lost. One possible explanation for these apparently opposing results is that in the present experiments we may have used lower amounts of trypsin, which could affect primarily other polypeptides (e.g. those of low molecular weight shown to be affected in Fig. 2, channels 6 and 7) that contribute to the formation of the ribosome-binding site (cf. Ref. 23) but may not directly interact with the ribosome. The approach used here (trypsin treatment) is essentially indirect, whereas Kreibich et al. (8--10) have used more direct approaches such as cross-linking and blockade of ribosome-binding by means of specific antibodies prepared against these polypeptides. In addition, the conversion experiments utilized here suffer from the serious limitation that trypsin may also attack ribosomal polypeptides involved in the ribosome-membrane interaction, so that detachment may be due at least in part to modification of the ribosomal components of the binding site. In fact, evidence of digestion of several ribosomal polypeptides was observed at a concentration of trypsin less than that used to effect conversion (cf. Fig. 2, channel 8). Another observation made in the present study pertinent to the possible involvement of these particular membrane polypeptides in ribosome binding was the variation in pattern noted among the ER membrane fractions of the various species studied (cf. Fig. 5). Our results indicate that the amounts of these two polypeptides show considerable variation
among rat, guinea pig, and rabbit liver ER membrane fractions. This suggests, assuming that these particular polypeptides are in fact constituents of the ribosome-binding site (8–10), that there may be intraspecies differences among the polypeptide components of this site. This possibility is subject to investigation by approaches such as cross-linking.

A number of novel findings resulted from the in vivo and in vitro studies of phosphorylation of rat and mouse liver ER membranes. No previous electrophoretic analyses of rat or mouse liver ER membranes labeled by the in vivo administration of sodium [32P]phosphate have been reported. Our studies have shown (i) that over 20 polypeptides in both RER and SER membranes are apparently phosphorylated in vivo, (ii) that the overall pattern of phosphorylation in vivo differs from that obtained in vitro, with for example one polypeptide being prominently labeled in vivo but not in vitro (cf Fig 6, channel 6), (iii) that minor differences between the patterns of phosphorylation of RER and SER membranes are detectable.

Two previous studies on in vitro phosphorylation of rat liver ER membranes have been reported: Jergil and Ohlsson (15) described two to three major phosphorylated polypeptides in RER and SER membranes, respectively, and Sharma et al. (16) reported some five major phosphorylated polypeptides in the latter membrane. A small number of additional minor phosphorylated polypeptides were apparently also observed in both of these studies. In the present study we have resolved some 25 phosphorylated polypeptides in both rat and mouse liver ER membranes after in vitro phosphorylation. We attribute the appreciably greater number of phosphorylated polypeptides detected in this study to three factors: (i) use of an electrophoretic system of relatively superior resolving power, (ii) use of relatively high specific activity [γ-32P]ATP for the in vitro incubations, (iii) use of a sensitive radioautographic method of detection. The in vitro patterns of phosphorylation of stripped RER and SER membranes were rather similar, although three consistent differences were observed – two additional components in the RER fraction and one in the SER fraction. Both this and the two previous studies (15, 16) have failed to find an effect of cyclic AMP on endogenous phosphorylation of ER membrane polypeptides. However, in the studies reported here we have utilized only one fixed concentration of each of ATP, cyclic AMP, and Mg2+; consequently, we are at present investigating the effects of using different ratios of these components on the phosphorylation patterns of the ER membranes. Recently Jergil et al. (24) have claimed that cyclic AMP may affect the in vitro phosphorylation of ER membrane polypeptides and that this may have some regulatory significance.

Several lines of evidence are compatible with our interpretation that the great majority of the phosphorylated components observed after electrophoresis and radioautography are indeed polypeptides. These include the susceptibility of all the bands to digestion by trypsin and the absence of effects of RNase, DNase, and extraction with lipid solvents on the observed patterns. Moreover, both labeled phosphoserine and phosphothreonine were detected in hydrolysates of stripped RER and SER membrane fractions labeled either in vitro (cf. Refs. 15 and 16) or in vivo. However, two other unidentified minor components were also observed in addition to these compounds. This raises the possibility that other amino acids may be phosphorylated, a point at present under investigation. It is also possible that certain other unusual compounds (e.g. phospholipids covalently linked to proteins (cf. Ref. 25)) may be present in the ER membrane, particularly following phosphorylation in vivo. A definitive approach to this problem will require the isolation of individual labeled phosphopolypeptides and determination of the stoichiometry of their phosphate-containing constituents.

The results from the in vivo phosphorylation studies are of interest in that they indicate that phosphorylation of ER membranes is probably a physiological event. Studies are at present in progress to examine the turnover of the phosphate moieties of the individual phosphopolypeptides, as the studies described here were performed on samples taken at one fixed time point (2 h after injection of 32P). The differences of pattern noted between in vivo and in vitro phosphorylation suggest that the results of the latter may be at least in part artificial, reflecting for instance, accessibility of sites not normally exposed in vivo. Differences between in vivo and in vitro patterns of phosphorylation have been described for ribosomal polypeptides (26).

Another point of interest is that in both types of labeling, polypeptides migrating in the areas of the gels corresponding to the phenobarbital- and 3-methylcholanthrene-inducible cytochrome P-450 and P-450, species of rat liver were radioactive. Phosphorylation of these important microsomal membrane proteins has been postulated (27); our results suggest that this hypothesis may be correct.

Because of a previous report (14) that phosphorylated polypeptides of the RER membrane may be involved in ribosome binding, our present demonstration of several differences in pattern between RER and SER membranes is of interest. Investigations are at present underway to determine whether the additional phosphopolypeptides noted in the RER membrane following in vivo phosphorylation may be involved in this process. Elucidation of other possible functions of phosphorylated polypeptides in ER membranes (28) must await further study.

The principal thrust of the present work has been to attempt to resolve by electrophoresis and thus enumerate the polypeptides of RER and SER membranes. Another objective has been to assist in defining the nature of the ribosome-binding site on the RER membrane. The major conclusions from the first line of study are that both ER membranes exhibit a complex polypeptide pattern and that there are several consistent differences in polypeptide pattern between them. The studies on phosphorylation, particularly those performed in vitro, indicate that this is a common modification of many of the polypeptides of both ER membranes. The experiments directed toward elucidating the nature of the ribosome-binding site are evidently indirect, correlative in nature, and consequently inconclusive with regard to interpretation. Several common polypeptides have been shown to be affected by treatments with concentrations of trypsin that markedly affect the capacity of RER membranes to bind ribosomes in vitro and that also result in detachment of ribosomes from the RER membrane. If the ribosome-binding site is polypeptide in nature, it is possible that at least some of the polypeptides noted here to be affected by trypsin are involved in the formation of this site. It would appear that cross-linking, immunological, and other approaches may afford a more direct resolution of the ribosome binding site. However, it is reasonable to assume that results from studies using an apparently more direct approach should harmonize with the results obtained here using modification of in vitro binding and ribosome detachment by trypsin, species comparisons, and phosphorylation to delineate any RER membrane poly-
peptides that may be specifically involved in ribosome binding.

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