EFFECT OF RIBOSOME STRIPPING PROCEDURES ON ANTIGENICITY AND CONFORMATION OF ENDOPLASMIC RETICULUM MEMBRANE[1]

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SUMMARY

The effects of 3 different procedures for stripping ribosomes from membranes on theantigenicity and conformation of isolated rough and smooth endoplasmic reticulum from rat liver were examined by microcomplement fixation and circular dichroism. Some of the blocked antigenic binding sites in rough endoplasmic reticulum became available after stripping of ribosomes. None of the 3 methods used is capable of stripping ribosomes completely from rough endoplasmic reticulum without the concomitant removal of protein from the membrane. Such loss of membrane protein by the stripping treatments is probably involved in the observed changes in rough endoplasmic reticulum, since a marked reduction in complement fixing capacity and in ellipticity of circular dichroism is observed also in smooth endoplasmic reticulum after similar treatments.

INTRODUCTION

During a study on the localization of a preneoplastic antigen in RER[2] and SER of putative premalignant hepatocytes (1) it was observed that RER was negative by immunodiffusion assay for one antigen, but became positive after stripping off the ribosomes. This finding stimulated our interest in studying the possible effects of such stripping on the conformation of liver cell ER membrane. Three different techniques were used to remove ribosomes (2-4) and the consequences of each on antigenicity and on ellipticity of circular dichroism were measured. Using antiserum against SER with microcomplement fixation and CD measurements, our results indicate that no single method of stripping ribosomes from ER, as commonly used, does so without concomitant removal of proteins from the membrane accompanied by obvious conformational changes.

MATERIALS AND METHODS

Fischer male rats (130-150 gm, Charles River, Wilmington, Mass.) were used. Liver fractionation and separation of RER, SER and free polysomes was done by the method of Sunshine et al (5). Purity of RER and SER was examined by elec-

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[2] Abbreviations: RER and SER - rough and smooth endoplasmic reticulum; CD - circular dichroism; EDTA - ethylenediamine tetraacetic acid.

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tron microscope as reported recently (1). The immunization schedule to induce antibody against isolated SER in New Zealand white rabbits was reported previously (6). Microcomplement fixation was performed according to Levine and Van Vunakis (7). CD was measured using a Jasco Model SS-20 for ORD with a modification for CD. All experiments were carried out at 23°C in nitrogen atmosphere in fused quartz 1-cm cells. Other experimental conditions for CD measurement were the same as reported previously (8). The CD-spectra for RER and SER were corrected and reconstructed according to the method described by Urry et al (9). Three stripping methods were chosen for this study: Method I by KCl-EDTA (2); Method II by puromycin-KCl (3); and Method III by pyrophosphate -citrate (4). Protein and RNA were determined by the method of Lowry et al (10) and Fleck and Munro (11) respectively.

RESULTS AND DISCUSSION

As is evident from Table 1, all 3 methods removed from over 60% to almost 90% of the RNA and from 49% to 64% of the protein from RER. Since the latter consists of protein from both ribosomes and membranes (Table 1) it is difficult to attribute any chemical alteration to the membrane alone. However, with SER containing no ribosomes, except by contamination, by definition, the bulk of the protein removed obviously is membrane-associated. Method III is the most efficient for removing ribosomes, but is also the most drastic for removing proteins. Since methods I and II have been used by two laboratories with only a single 'extraction' (2,3), this procedure was followed by us also.

Figure 1A clearly shows that the unstripped SER required only 1μg of protein to obtain the maximal complement fixing capacity (85%) following reaction with anti-SER antiserum, whereas the complement fixing capacity of unstripped RER was as low as 5%. Stripping of RER by different methods uncovered antigenic binding sites to different degrees. Stripping by method III, removing 89% of ribosomes from RER, revealed only 50% of complement fixing capacity at an antigen concentration of 2μg. In contrast, after stripping by method II, with removal of 50% of ribosomes, the complement fixing capacity was as high as 72% at the same antigen concentration. Stripping by method I, with 63% of ribosomes removed, uncovered slightly higher antigenic binding sites than that by method III. Thus, the degree of uncovering of antigenic binding sites by various stripping treatments is not proportional to the percentage of ribosomes removed. These results suggest that stripping has an effect on antigenicity additional to that of removing ribosomes. This is also reflected in the loss of membrane proteins from RER caused by stripping (Table 1). To test this possibility, SER was subjected to the same stripping treatment by the very same 3 methods and microcomplement fixations were done. As shown in Table 1, the amount of protein removed from SER in each stripping method was essentially the same as that from RER by the same method. Figure 1B shows that the complement fixation curves of stripped SER after the 3 different stripping treatments were altered, as compared to that of unstripped SER. In comparison

TABLE 1: Effect of various stripping methods on the constituents of ER membrane[3]

Stripping treatment	mg RNA/2ml	% RNA removed[4]	mg Protein /2 ml	% Total protein removed[4]	% Protein removed as ribosomal protein[5]
Control:					
RER	1.79±0.059		3.16±0.017		
SER	0.38±0.012		4.88±0.138		
Method I:					
RERst[6]	0.65±0.025	63.4±3.8	1.52±0.04	51.9±2.6	31.3
SERst	0.12±0.011	67.8±9.2	2.04±0.067	58.2±3.3	4.1
Method II:					
RERst	0.43±0.022	76.2±5.1	1.60±0.058	49.4±3.6	39.2
SERst	0.10±0.003	74.4±3.0	2.56±0.045	47.5±1.8	5.4
Method III:					
RERst	0.18±0.012	89.9±6.7	1.14±0.039	64.1±3.4	35.9
SERst	0.06±0.003	83.9±5.0	1.76±0.033	63.9±1.9	4.6

[3] Isolated RER and SER from normal liver were subjected to the various stripping treatments.

Method I: by KCl-EDTA (2), isolated ER membranes were dialyzed at 4°C overnight against 200 volumes of 50mM Tris-HCl, 25mM KCl and 5mM EDTA, pH 7.5. Method II: by puromycin-KCl (3), isolated ER membranes were suspended in 0.25M sucrose, 2.5mM $MgCl_2$, 0.05M Tris-HCl, containing 0.5M KCl and 5 x 10^{-4}M puromycin, pH 7.6 and incubated at 0°C for 1 hour followed by 10 min at 25°C. Method III: by pyrophosphate-citrate (4), membranes were suspended in stripping solution containing 0.25M sucrose, 80mM Na-citrate, 10mM Na-pyrophosphate and 10mM Na-phosphate (monobasic), pH 7.4 and stood at 0°C for 30 min with occasional stirring. At the end of incubation, membranes were harvested by centrifugation at 105,000g in a Beckman 60 Ti rotor for 45 min. This stripping procedure was repeated 3 times. At the end of each stripping treatment membranes were collected by centrifugation and suspended in 1.2M sucrose TKM (50mM Tris-HCl, pH 7.5, 25mM KCl and 5mM $MgCl_2$) layered over 2M sucrose TKM and spun at 105,000g for 4 hours to separate the stripped membranes and detached ribosomes. The membrane-containing bands at the interface were aspirated, diluted with 2-3 vols. of TKM without sucrose and harvested by centrifugation. The control RER and SER were suspended in 0.25M sucrose TKM and subjected to the same centrifugation forces without prior stripping treatment.

[4] Mean ± standard deviation of 4 experiments.

[5] % Ribosomal protein was calculated based on RNA/protein of 1.22 of isolated ribosomes.

[6] Stripped RER and SER are designated as RERst and SERst.

to 1μg of protein required for unstripped SER to obtain the maximal complement fixing capacity (85%), stripped SER required more than 2μg of protein to reach the same capacities. The requirement of a higher concentration of ant-

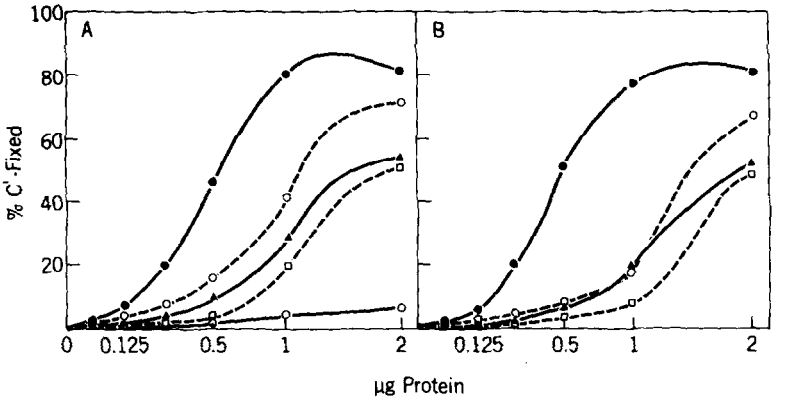

Figure 1: Micro-complement fixation of ER membranes isolated from normal rat liver against anti-SER antiserum (1:1,600).
A. Unstripped SER (●—●) and RER (o—o); stripped RER by method I (▲—▲); method II (o---o); and method III (□---□).
B. Unstripped SER (●—●); stripped SER by method I (▲—▲); method II (o---o); and method III (□---□).

igen for maximum fixation observed in 3 stripped SER preparations indicates the reduced affinity of a majority of the antigenic determinants (12). The lateral shifts of complement fixing curves of stripped SER suggest a conformational change of the membrane constituents (13). Among the 3 stripping methods used, method III caused the greatest effect on the antigenicity, as judged from the complement fixing curve. In comparing the amount of ribosomes and protein removed both from stripped RER and SER (Table 1) with the complement fixing curves (Figures 1A and 1B) in each stripping treatment, it appears that complement fixing capacity is increased by procedures that remove ribosomes in stripped RER but decreased in stripped SER. These findings are in agreement in principle with that of Stratman et al (14), who reported that stripping of rat liver SER and RER reduced by 15% and increased by 30% respectively, the sulfhydryl groups available for carboxamidemethylation by iodoacetamide.

As shown by Osler and Hill (15), the complement fixing capacity of rabbit immune systems depends on the affinity of antibody for its antigen and the subsequent complex formation. Since microcomplement fixation assay employs antiserum and antigen at very low concentrations (1:1600 dilution for antiserum and 2µg protein in 6ml reaction mixture down to 0.0625µg in these studies) it is likely that only the antibodies of highest affinity are being measured. A change which alters the complementarity between one of the antigenic determinants on the protein and its specific antibody may also decrease the affinity

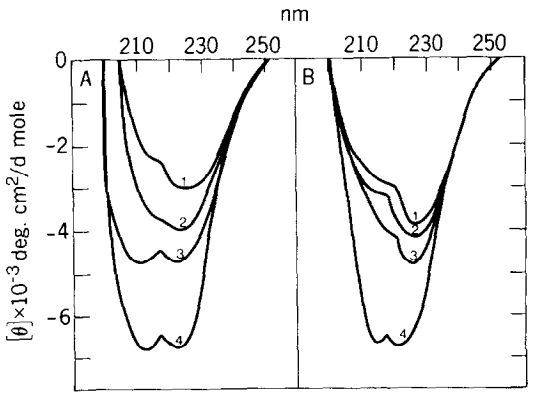

Figure 2: Circular dichroism spectra of ER membranes of normal rat liver at wavelengths between 200-250nm. CD spectra were measured at protein residue concentrations of 10μg/ml for all cases. The ellipticity [θ] of the membranes is expressed in degree centimeters squared per decimole of amino acid residue, using a mean residue weight of 115 for membrane amino acids.
A. Curve 1, stripped RER by method III; curve 2, stripped RER by method I; curve 3, stripped RER by method II; curve 4, unstripped RER.
B. Curve 1, stripped SER by method III; curve 2, stripped SER by method I; curve 3, stripped SER by method II; curve 4, unstripped SER.

between them. Thus, even a local conformational change in only one of several antigenic determinants may be magnified during the process of antigen-antibody complex formation and be sufficient to alter the complement fixation curve.

The effect of stripping on membrane conformation was further examined by circular dichroism. As shown in Figure 2, the spectra of unstripped RER (Figure 2A, Curve 4) and SER (Figure 2B, curve 4) are similar in shape and amplitude. Both have well-defined minima at 210 and 222 nm, with an ellipticity of around -6,800 deg cm^2/d mole. However, after stripping the ellipticities were markedly decreased in both cases. Among the stripping methods used, method III (Curve 1, both in Figures 2A and 2B) exhibits the greatest effect, then method 1 (Curve 2), followed by method II (Curve 3), as judged by the reduction in ellipticity. It is interesting to note that stripping of RER by method I (Figure 2A, Curve 2) and III (Curve 1) caused a red-shift of 210-nm-peak and formed a shoulder at about 215 nm. In addition, the crossover point also shifted from 200 to 205 nm. The nature of this shift is not known. The same direction of effect on the CD spectrum of stripped SER as of stripped RER was observed (Figure 2B). The shape of the spectra obtained after stripping by method I, II and III was not significantly different. However, a red-shift of 210-nm-peak was observed in all cases.

These findings clearly indicate that the stripping methods currently used for removing ribosomes from RER concomitantly remove membrane protein and cause the subsequent effect on antigenicity and conformation. None of the 3 methods used is capable of removing ribosomes without losing membrane protein. Thus, caution must be taken in studying the *in vitro* reconstitution of polyribosomes by allowing the interaction of removed ribosomes with stripped RER. In view of the observations presented in this communication and those reported by Hochberg et al (16), the biological validity of the reconstitution of RER using ^{32}P-labelled polyribosomes and stripped RER (17) might have to be re-evaluated.

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