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Coagulation factor III (tissue factor) interaction with phospholipid vesicles induced by cadmium: characterization of the reconstituted protein-membrane complex

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Coagulation factor III (tissue factor) is a membrane glycoprotein which serves as a cofactor in the proteolytic activation of factor X and factor IX by factor VIIa. Mixing of human placental factor III apoprotein with vesicles of bovine brain phospholipids does not produce significant reconstitution of factor III activity, but, when the mixture of apoprotein and vesicles is made 5 mM with $CdCl_2$, the apoprotein is incorporated into the vesicles. Ultracentrifugation on sucrose density gradients demonstrated that the active factor III-lipid complex formed by reconstitution with vesicles had a density indistinguishable from that of the complex formed by detergent dialysis. Vesicles isolated after centrifugation were shown to range in diameter from 20 nm to over 100 nm using the electron microscope. Gel filtration showed that factor-III activity was associated with all size-classes of vesicles. The presence of factor III activity in the smallest vesicles argues for a specific cadmium-mediated reconstitution of the apoprotein with phospholipid vesicles.

Coagulation factor III (tissue factor), present in the membrane or reconstituted into phospholipid vesicles, accelerates the proteolytic activation of both factor X and factor IX by factor VIIa (1,2,3). The assembly of factor III into phospholipid vesicles during deoxycholate dialysis is promoted by $CdCl_2$ (4), and although the apoprotein does not interact significantly with preformed phospholipid vesicles, it can be induced to do so by the addition of $CdCl_2$ (5). The control of factor-III activity is not well understood but is known to be affected by the particular phospholipids bound to the apoprotein (1,6-9) and by the amount of lipids present (10). This report demonstrates the cadmium-induced incorporation of human placental factor III into preformed phospholipid vesicles and describes the resultant protein-membrane complexes.

Experimental Procedures

Factor III was prepared from heptane-butanol-extracted acetone powders of human placentas obtained at parturition (11). The factor III was extracted with Triton X-100, followed by ammonium sulfate precipitation, and chromatography on Phenyl-Sepharose, Con A-Sepharose, and DEAE-cellulose (5). Factor-III activity was determined with the two-stage clotting assay described by Pitlick and

Nemerson (11). The final factor-III preparation had a specific activity of 1.7 x 10^5 units/mg, which is a 2000-fold purification from the acetone powder (assuming 100% extraction with the Triton X-100). We estimate that a 100 000-fold purification will be required to achieve homogeneity.

Factor III-membrane reconstitution by deoxycholate dialysis was accomplished as described elsewhere (4,5). For incorporation of factor III into preformed vesicles, mixed phospholipids from bovine brain (11) dissolved in 0.25% sodium deoxycholate at 2.5 mM were placed in a conical microcentrifuge tube, covered with dialysis membrane, and floated on 0.05 M Tris, 0.1 M NaCl, 0.001% NaN_3, pH 7.6, overnight at 4°C. Phospholipid concentration was determined by phosphate analysis as described (11,12). The reconstitution mixture (final volume 0.5 ml) contained 50 µl of 0.5 M Tris, 1 M NaCl, 0.01%

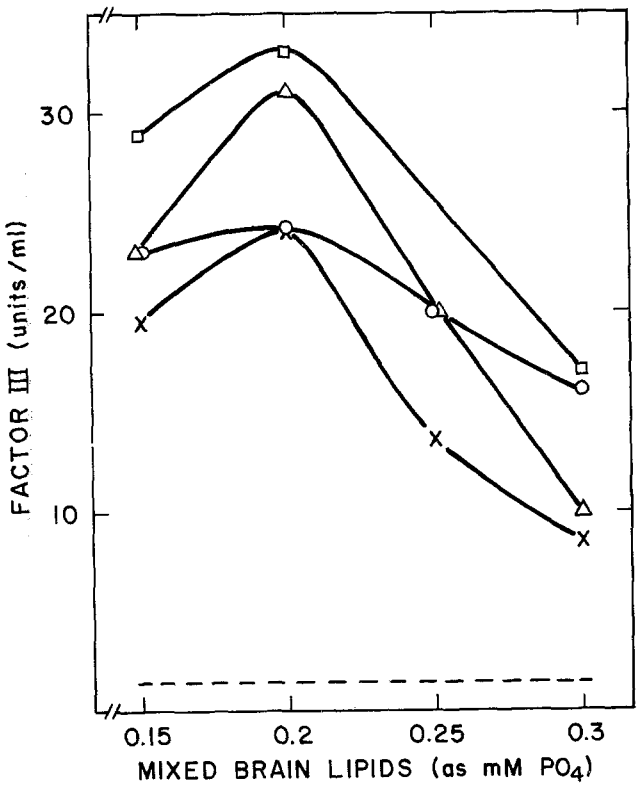


Fig. 1. Determination of optimum vesicular lipid concentration for reconstituting factor-III activity. Bovine factor X and factor VIIa for the two-stage assay were provided by Dr. Yale Nemerson. Clotting times from a typical standard curve for this series of experiments were 17 sec for 100 units/ml and 92 sec for 1 unit/ml. Factor III was reconstituted with lipid vesicles without $CdCl_2$ (----) and with $CdCl_2$ concentrations of 2 mM (x——x), 3 mM (△——△), 4 mM (□——□), and 6 mM (○——○).

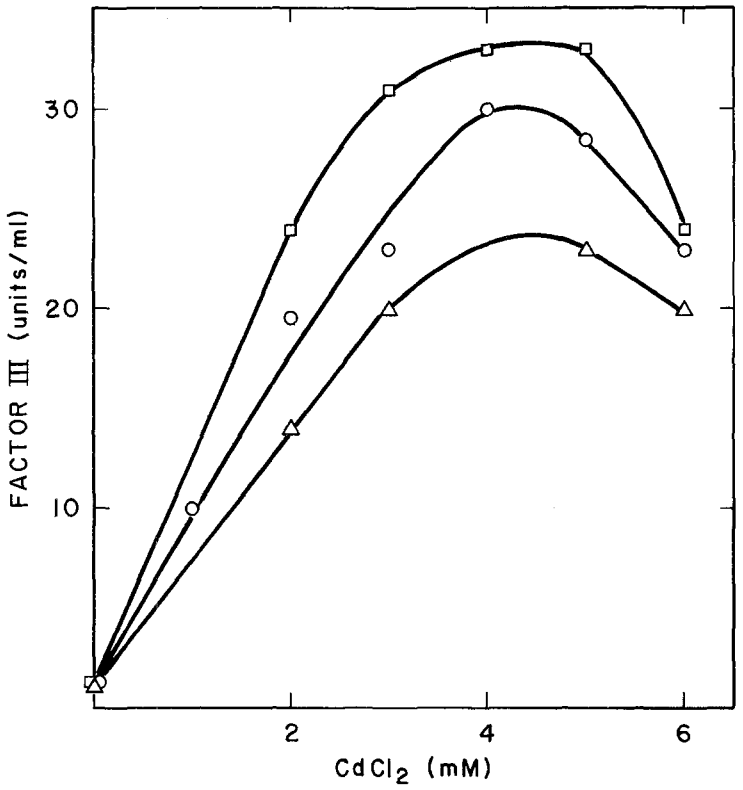


Fig. 2. Determination of the optimum concentration of $CdCl_2$ for promoting reconstitution of factor-III activity. Vesicle concentrations (determined as lipid phosphate) of 0.15 mM (○——○), 0.2 mM (□——□), and 0.25 mM (△——△) were used.

NaN_3, pH 7.6; 10 µl of the factor-III preparation (0.38 µg of protein); and test amounts of the dialyzed phospholipids. After 10 min at 24°C, $CdCl_2$ was added from a 50 mM stock to give a final concentration of 5 mM cadmium (or test concentrations as indicated). Coagulation assays were conducted after an additional 10 min at 24°C.

Results

Early experiments (1) had shown that factor III bound phospholipids in the presence, but not in the absence, of deoxycholate. Experiments described here have confirmed that mixing factor-III apoprotein with preformed vesicles does not restore factor-III activity. Addition of $CdCl_2$ to the mixture, however, promotes the interaction of factor III and phospholipid vesicles, which restores factor-III activity. Optimum concentrations for restoration of activity were determined for lipid vesicles (Fig. 1) and for $CdCl_2$ (Fig. 2). Freshly prepared stocks of lipids and $CdCl_2$ increased the reproducibility of their respective optima. The optimum concentrations of lipid and $CdCl_2$ were independent of each other (Figs. 1 and 2), and the $CdCl_2$ optimum

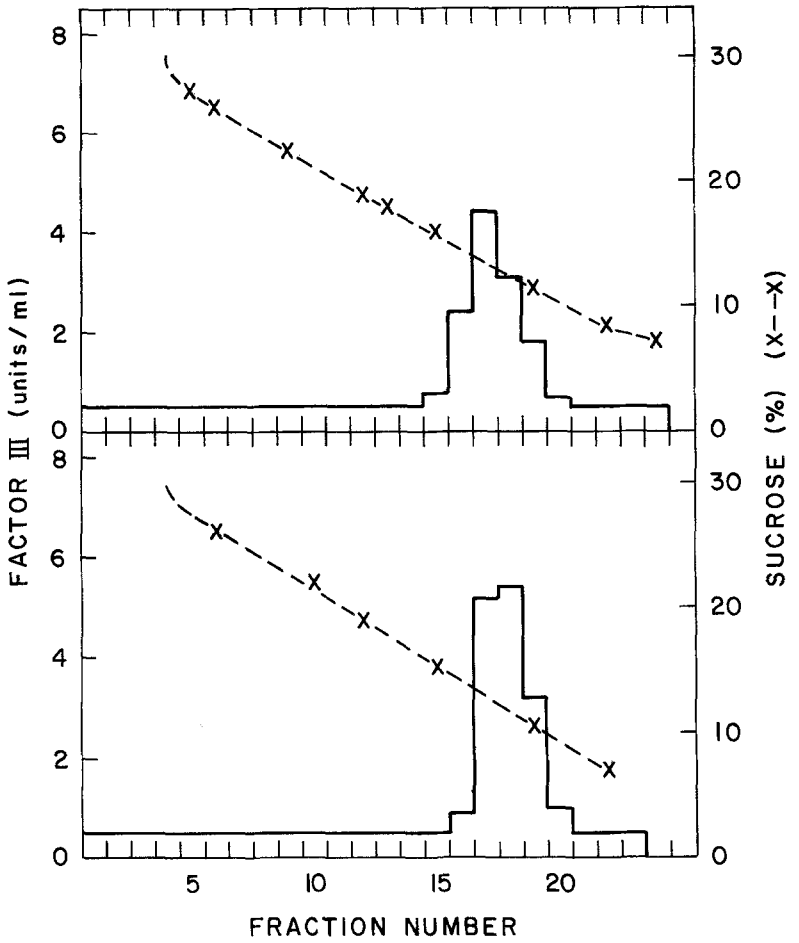


Fig. 3. Sucrose-gradient fractionation of factor-III complexes. Ultracentrifugation was conducted as previously described (4) except that factor-III sample volumes were 300 µl and the centrifugation was conducted at 40 000 rev/min at 20°C for 22 h. The sucrose gradient contained 0.05 M Tris, 0.1 M NaCl, 0.001% NaN_3, pH 7.6. The factor-III samples compared were relipidated before centrifugation by deoxycholate dialysis with $CdCl_2$ (lower panel) and by $CdCl_2$-mediated assembly into preformed vesicles (upper panel). The gradient fractions were assayed directly for factor activity.

was independent of the amount of factor-III apoprotein. When the apoprotein concentration was increased 2.5-fold, the optimum $CdCl_2$ concentration remained near 5 mM. The lipid optimum, however, was increased to 0.5 mM, demonstrating that the factor-III activity recovered by assembly into preformed vesicles is dependent on the ratio of phospholipid to protein (211 to 1 [w/w] at the optimum in these experiments), as had been found for reconstitution by detergent

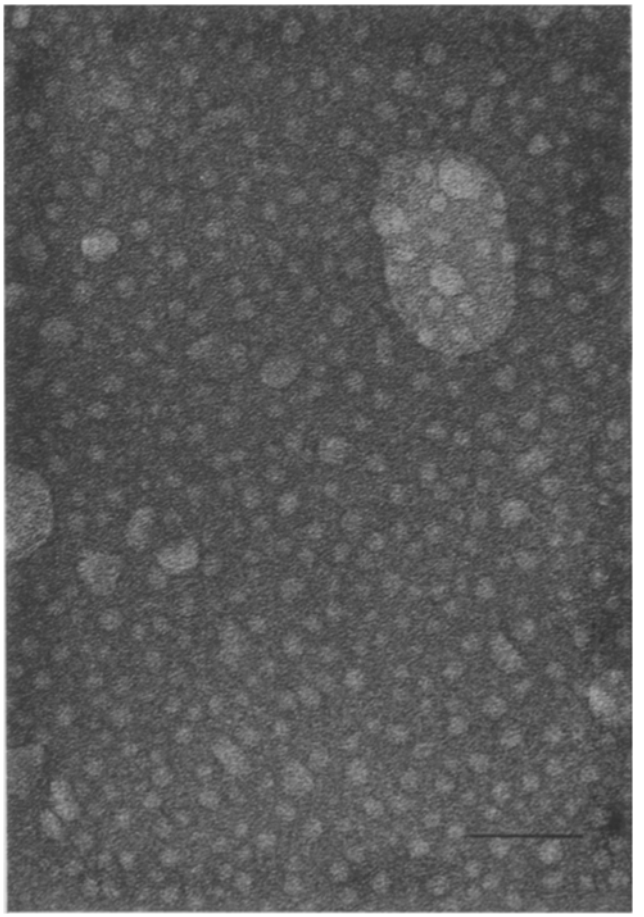
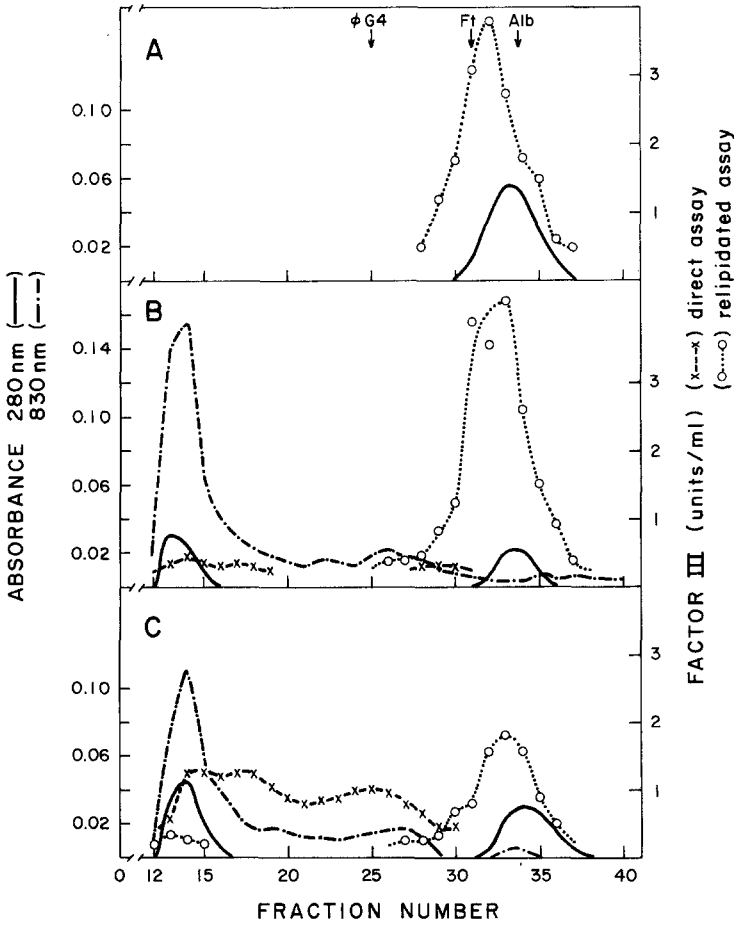


Fig. 4. Electron micrograph of factor-III complexes. The vesicles contained in the peak factor-III fraction (number 17) after ultracentrifugation of the apoprotein assembled into preformed vesicles (Fig. 3, upper panel) are shown. The vesicles were allowed to settle on carbon-coated grids, were stained with 1% phosphotungstic acid/0.05 M Tris, and were examined with a Philips EM300 at 80 kV. The bar corresponds to 100 nm.

dialysis (5,8,12). In similar experiments using $CaCl_2$ and $MgCl_2$, $CaCl_2$ (at 20 mM) produced a 3-fold increase in factor-III activity, considerably less than the increase seen with $CdCl_2$.

The reconstituted factor III prepared using preformed vesicles was compared with factor III reconstituted by deoxycholate dialysis (with added $CdCl_2$) by ultracentrifugation on sucrose density gradients. Both methods for reconstituting factor-III apoprotein with phospholipid vesicles produced active complexes which banded between 13 and 16% sucrose (Fig. 3). Fraction 17 of the sample reconstituted from preformed vesicles contained vesicles with diameters ranging from about 20 nm to more than 100 nm as revealed by electron microscopy (Fig. 4).

Reconstituted factor III was gel-filtered on Bio-Gel A15m to determine which size-classes of vesicles were associated with factor-III activity. For comparison, factor-III apoprotein alone and mixed with vesicles without $CdCl_2$ were also gel-filtered. Direct coagulation assays of the column fractions detected significant factor-III activity only in fractions containing vesicles reconstituted in the presence of cadmium (Fig. 5). The factor-III activity was present in fractions eluted at the excluded volume and in all subsequent fractions through those in which bacteriophage G4 (diameter 28 to 30 nm [15]) had eluted during calibration of the column. The relative factor-III activity which eluted at the excluded volume and in subsequent fractions varied quantitatively among experiments, but the demonstration of factor-III activity associated with all vesicle sizes was a reproducible finding. No factor-III activity was detected in fractions which eluted after the vesicles until further relipidation was carried out to reveal the presence of factor-III apoprotein which had not been reconstituted in the initial reaction. The apoprotein eluted just prior to albumin, even in the presence of phospholipid vesicles (Fig. 5A, B).



Discussion

The cadmium-promoted assembly of factor-III apoprotein into preformed phospholipid vesicles is an unexpected observation, since the apoprotein does not interact appreciably with phospholipid vesicles (1,5) and is not significantly induced to do so by $CaCl_2$ or $MgCl_2$. The vesicle-reconstituted factor III-lipid complex is very similar to the active complex formed by deoxycholate dialysis. Both modes of reconstitution have a $CdCl_2$ optimum near 5 mM, show optimum lipid concentrations at similar lipid-to-protein ratios (4,5), and produce factor-III vesicles with indistinguishable densities on sucrose density-gradient ultracentrifugation. The persistent association of factor III reconstituted with vesicles using $CdCl_2$ during prolonged centrifugation and on gel filtration in the absence of $CdCl_2$ demonstrated that the induced association is not readily reversible. Electron microscopy revealed that the sizes of the vesicles in a single sucrose density fraction with factor-III activity spanned the range of diameters from about 20 nm to well over 100 nm. The 20-nm vesicles are reminiscent of those observed by Huang after sonication and gel filtration of phosphatidylcholine vesicles on Sepharose 4B (16).

Fig. 5. Sizing of reconstituted vesicles by gel filtration. A column (1.7 x 20 cm) of Bio-Gel A15m, equilibrated in 0.05 M Tris, 0.1 M NaCl, 0.001% NaN_3, pH 7.6, was allowed to flow by gravity at approximately 15 ml/hr. Fractions were collected at 4-min intervals. Factor-III samples were prepared for gel filtration using 25 µl of the apoprotein with the appropriate increase (2.5-fold) of phospholipids to produce optimum reconstitution (see 'Results'). Phospholipid in the fractions was estimated according to Bartlett (13). Fractions were assayed directly for factor-III activity, and were then individually reconstituted and assayed again to detect any apoprotein which had not been reconstituted in the initial reaction. Relipidation of the individual fractions was done by combining 100 µl of each fraction with 25 µl of mixed lipids in 0.25% sodium deoxycholate, 10 mM Tris, pH 7.5. After 10 min, 12 µl of 50 mM $CdCl_2$ was added. The samples were assayed for coagulant activity after 10 min. During calibration of the A15m column, the elution of bacteriophage G4 was monitored by assaying fractions for plaque-forming ability on *E. coli* C (14). The preparations presented were (A) factor-III apoprotein alone, (B) factor-III apoprotein mixed with phospholipid vesicles, and (C) factor III mixed with phospholipid vesicles and made to 5 mM $CdCl_2$ for reconstitution. Reference standards were bovine serum albumin (Alb), horse-spleen ferritin (Ft), and bacteriophage G4 (ϕG4).

The demonstration that calcium, which effectively promotes vesicle-vesicle fusion (17-19), does not promote reconstitution of factor-III activity nearly as well as does cadmium, suggested that the assembly of the apoprotein into the lipid bilayers involves a specific interaction rather than random incorporation during vesicle-vesicle fusion. Confirmation of this interpretation, however, required the demonstration of factor-III activity associated with the 20-nm vesicles. Gel filtration on Bio-Gel A15m revealed that factor-III activity was indeed associated with the smallest vesicles observed as well as with the larger lipid structures. Although factor-III activity may become associated with the larger vesicles by incorporation into the 20-nm vesicles followed by vesicle-vesicle fusion, the cadmium-mediated apoprotein-vesicle assembly mechanism may operate not only with the 20-nm vesicles but with the more extensive membranes observed as well. As previously discussed (5), the singular ability of cadmium to promote factor III-vesicle assembly may be the result of cadmium chloride complexes rather than the divalent cation. Indeed, several preliminary experiments have indicated that the ratio of cadmium to chloride is more important for reconstitution of factor III-vesicle complexes than is the absolute concentration of cadmium.

These experiments have led to a rapid (20-min) method for reconstituting factor-III activity from the apoprotein and phospholipid vesicles, and demonstrated that cadmium can induce interaction of the apoprotein with vesicles. The centrifugation and gel filtration results can be used for preparation of factor III-vesicle complexes of defined size and lipid-to-protein ratio. This is especially important for studies of factor-III activity, since factor VIIa (the enzyme for which factor III serves as a cofactor) and factor X (the substrate) both bind to phospholipid surfaces (20), and this may help explain data obtained in studies of factor-X activation kinetics (Dr. Yale Nemerson, personal communication). Excess phospholipids inhibit the activation of factor X, possibly by sequestering factor X on vesicles which contain no factor III (10). Factor-III apoprotein not incorporated into vesicles may also affect the activation of factor X by retaining functional enzyme or substrate binding sites. Additionally, factor-X activation may require binding of factors X and VII to vesicles containing factor III. The reaction could therefore be influenced by geometric constraints imposed upon the lipid-cofactor-enzyme-substrate complex by the size or curvature of the lipid surface on which these components interact. The influence of vesicle size and structure can be illustrated by emphasizing that the external phospholipid surface available for these reactions is approximately 50% greater in a system containing only 20-nm vesicles than in a system containing larger vesicles in which the bilayer approaches a planar geometry (21). The spacing of lipid head groups, packing of acyl chains, and the asymmetry of lipid distribution across the bilayer are also influenced by vesicle size (21). These considerations demonstrate the importance of physically defined factor III-containing vesicles with which to investigate further aspects of factor-III activity and its regulation.

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